University of Bath

PHD

The function of differentially methylated region one and its effect on insulin-like growth factor two expression

Kelly, Sharon

Award date:
2003

Awarding institution:
University of Bath

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 13. May. 2019

The function of differentially methylated region one and its effect on insulin-like growth factor two expression.

Submitted by Sharon Kelly
for the degree of PhD
of the University of Bath 2003.

Copyright

Attention is drawn to the fact that copyright of this thesis rests with the author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with the author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University of Bath and may be photocopied or lent to other libraries for the purposes of consultation.

Signed: Sharon Kelly

Date: 5/3/04

UMI Number: U174794

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U174794
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF BATH
20 APR 2004
Ph.D.

Acknowledgements

I would like to thank Dr. William Bennett for his help in teaching me how to carry out transgenesis injections and for his help in all other matters molecular. Also Dr. Ghislaine Dell for her help with transient transfections and for building the constructs. Dr. James Dutton for his proof reading and his input with the transfections and for his help with all things tissue culture related. Dr. Marika Charalambous for her help with transgenics and all things to do with imprinting and Igf2 specifically. Thanks to Tia Smith for her help with Grb10 information and p53 mouse weighing, Treve Menheniott for the hepG2 cells and for his help in histology and for the picture of Christian, Dr. Tracey Crewe for her help with statistics and all things associated with p53 and tumours, everyone in 5 West for their help over the years and Chris Apark in histology for his tumour sections. Thanks to the pathologist for his tumour diagnosis, Wolf Reik for the DMR1 deletion mice and Alan Clarke for the p53 knock-out mice.

I would also like to thank my supervisor Dr. Andrew Ward for all his help, especially in surgery every Thursday over the last few years.

Last but not least my thanks to everyone in Lab 0.76 for making it a great place to work and for leaving me with some very happy memories. Thanks a million everyone.

This PhD was funded by the University Of Bath.

This is dedicated to my family, Edward, Joan, Gillian and Christine, the monster has been laid to rest.
Thank you for everything.

Abbreviations

Air	*Antisense insulin-like growth factor two receptor transcript.*
As	Angelman Syndrome.
BSA	Bovine serum albumin.
BWS	Beckwith Weidemann syndrome.
CCD	Centrally conserved domain.
CTCF	CCCTC-binding factor.
DMD	Differentially methylated domain.
DMR	Differentially methylated region.
DNA	Deoxyribonucleic acid.
Dnmt	*De novo methyltransferase.*
E14.5	Embryonic day 14.5.
FBS	Foetal bovine serum
FISH	Fluorescence *in situ* hybridisation.
ICR	Imprinting control region.
Igf2	*Insulin-like growth factor two.*
Igf2r	*Insulin-like growth factor two receptor.*
kb	Kilo base
LacZ	Beta-galactosidase.
LOI	Loss of imprinting.
MeCP2	5' methyl-cytosine binding protein two.
PBS	Phosphate buffer solution.
PCR	Polymerase chain reaction.
PWS	Prader-Willi syndrome.
RNA	Ribonucleic acid.
SFM	Serum free medium.
UPD	Uniparental disomy.
UV	Ultra violet.

Table of contents

Introduction

1.1Genomic Imprinting

Diploid organisms inherit one set of genes from their mother and one from their father. The expression of a subset of these genes however depends on which parent they were inherited from. This is known as genomic imprinting. Mendelian inheritance is when both alleles in a heterozygote organism are equally expressed. Imprinted genes do not follow the rule of Mendelian expression. Many organisms have a type of imprinting mechanism ranging from unicellular organisms (Matagne 1987), arthropods (Spofford 1976), mammals (Efstratiadis 1994) and angiosperms (Matzke 1993). In 2001 Reik et al. carried out experiments in which mouse embryos were created, in which both sets of chromosomes originated from parents of the same sex, showed differences in phenotype depending on whether the chromosomes came from the male or female (Reik 2001) . Genomic imprinting was discovered in the early nineties by creating mouse uniparental embryos and mouse embryos which had uniparental disomys (Cattanach 1985). Nuclear transplantation was used to create embryos that inherited chromosomes from one parent only. In the embryos that had two paternal sets of chromosomes (androgenetic) it was noted that the extraembryonic tissues such as the placenta were well developed but the embryo itself was not. In embryos that had two maternal sets of chromosomes (parthenogenetic or gynogenetic) it was noted that the embryo was well developed but the extraembryonic tissues were not. This is discussed more fully later (Section 1.2.). About 50 mammalian-imprinted genes have been identified (www.mgu.har.mrc.ac.uk) and it is estimated that the mouse genome may contain about 100 imprinted genes in total. Although imprinted genes are often referred to as being 'active' or 'silent' there is often some detectable RNA from the so called 'silent' allele. If the expression ratio of one allele to the other is less than 3:1 or greater it can be considered to be an imprinted gene (Burns 2001) . In the case of *Igf2* RNA expression from the 'silent' maternal allele is around 10% that from the paternal allele in the embryo apart from the biallelic expression in the leptomeninges (DeChiara 1991).

Later it was found that epigenetic marks on the genome differentiated the egg and sperm DNA leading to differential gene expression known as imprinting. Most known mammalian imprinted genes (80%) tend to be clustered and contain epigenetic markings. Epigenetic marks differentiate the maternal from the paternal allele without altering the

nucleotide sequence and are discussed more fully in Section 1.7. Many imprinted genes are found close to another, oppositely imprinted gene such as *Igf2* and *H19* on mouse chromosome 7, *Dlk1* and *Gtl2* on mouse chromosome 12. Groups of functionally related genes such as the Hox genes that arise by gene duplication events are often grouped together (Von Mering 2002) . In nematodes, it has been observed that co-regulated genes are sometimes organised into regulatory units called operons. This clustering of imprinted genes may make it easier for *cis*-regulatory elements to control the imprinting of a number of genes within a cluster (Caspary 1999) . However non-imprinted genes are also clustered and so this cannot be regarded as a defining feature of imprinted genes. The clustered genes may have areas of differential methylation or histone acetylation between the maternal and paternal allele, and may also have replication timing differences and chromatin structure differences (Brannan 1999) . Methylated DNA typically generates inactive chromatin structure and this can spread to neighbouring unmethylated DNA exerting its silencing effect over a larger portion of the chromosome. This mechanism of controlling gene expression may be one reason to cluster imprinted genes (Hu 2000).

Imprinted genes are usually rich in CpG islands, which are defined as a 200bp region of DNA with a G+C content of over 50%. Analysis of the mouse genome found that 47% of mouse genes are associated with CpG islands but this frequency increases to about 88% when mouse imprinted genes are examined. Direct sequence repeats are also found near to these CpG islands (Paulsen 2000). About 10% of the genome consists of repetitive DNA sequences mostly located in heterochromatic regions around the centromeres and telomeres. Mutations in these repeat sequences are associated with haemophilia and cancers of the breast and colon.

To imprint a gene epigenetic marks must be established. Imprinting marks are established in the sperm and egg and are maintained after fertilisation and throughout development (Table 1). Imprinting requires that the marks must be erased and reset correctly in the germ cells of the next generation. This erasure is associated with a genome wide demethylation of the DNA, which is completed by embryonic day 12-13 (E12-13) in the mouse. Then *de novo* methylation occurs with methyltransferase from the *Dnmt* genes re-establishing the imprint.

In mice lacking the *Dnmt1* gene *Igf2* is no longer expressed (Li, Beard et al. 1993) showing the importance of methylation in the control of expression of *Igf2* (Section 1.6.1).

However, a notable exception is the *Mash2* gene that's imprinted expression remains in *Dnmt1* knockout embryos (Dell 1997).

Other genes affected by a lack of methyltransferase are the *Xist* gene leading to partial inactivation of one of the X-chromosomes in females (Beard 1995), *H19* is biallelically expressed and maternal *Igf2r* is silenced (Li, Beard et al. 1993) .

1.2 Evolution of imprinting.

There are many theories to explain the existence of imprinted genes. Large proportions of imprinted genes are associated with fetal growth or cell proliferation. One theory is that the paternally expressed genes are in competition with the maternally expressed genes. The paternally expressed genes tend to act selfishly and opportunistically, with the aim of utilising maternal resources as they can so that the offspring will develop to be as fit as possible, even if this occurs at the expense of the other siblings (parent offspring conflict hypothesis)(Moore 1991).

The maternally expressed genes aim to reduce this competition between siblings by trying to allocate all of maternal resources equally but not to the extent as to endanger the future reproductive fitness of the mother (Moore 1991). Postnatally the competition effects of imprinted genes can also be observed. Mice with paternal disomys have been observed to be hyperkinetic and those with a maternal disomy were observed to be hypokinetic (Cattanach 1985). Female mice with a deficiency in the paternally expressed *Mest* gene shown abnormal maternal behaviour. These mice do not respond as normal to their newborn pups with 84% of litters having at least one unattended pup. Half of the pups were not properly cleaned after birth and were not fed properly. This implies a paternal control over the distribution of maternal resources not only during embryogenesis but postnatally also .

Experimental evidence from mice shows that in an androgenetic embryo, the embryo itself is underdeveloped and the extraembryonic membranes are well developed. Parthenogenetic embryos on the other hand have a well-developed embryo and the extraembryonic membranes are underdeveloped. These membranes (placenta) divert maternal resources to the relevant foetus at the possible expense of the other fetuses (Moore 1991). Two examples of genes that demonstrate the different priorities of the maternal and paternal genes are *Grb10* and *Igf2*. *Grb10* is a maternally expressed

imprinted gene. Disruption of this gene in mice leads to a 30% increase in size of the embryo and the placenta (Charalambous 2003). Without the maternal allele to keep the growth rate in check the embryos are larger. *Igf2* is a paternally expressed imprinted gene. Disruption of this gene leads to a 30% reduction in the size of the embryo (DeChiara 1990). Without the paternal allele to allocate maternal resources to the embryos the mice are smaller.

This theory fits in very well with organisms that are polyandrous. It can be applied both when different litters have different fathers and also when different fetuses within a litter have different fathers. However, imprinted genes have been found in monogamous species such as *P. polionotus* where they should not be necessary according to this theory. Nevertheless, it has been argued that such species may have evolved from polyandrous ancestors and that if the male dies the female will mate with another male and this may be sufficient to retain the imprinting mechanism.

Arguing against the parent offspring conflict hypothesis is X chromosome inactivation. This can occur in two ways, either the paternal X chromosome is inactivated (as occurs in the trophectoderm and primitive endoderm) or the X chromosome is randomly inactivated (as occurs in somatic tissues of female eutherians) (Moore 1991). It could be argued that it would be more beneficial to the paternally derived genes if all of them were active in the placenta. Also imprinting would be expected in somatic tissues and yet X chromosome inactivation is random.

Another theory regarding the existence of imprinting is that it works as a defence mechanism. It is thought to prevent the production of parthenogenic offspring that in the long term have a higher chance of becoming extinct and so are not beneficial to the total population. Since imprinting makes parthenogenic offspring non-viable it allows the mother to reallocate her resources to viable offspring (sexually produced). The parthenogenic offspring are aborted relatively early, in order to conserve maternal resources. However, this theory cannot explain the differential paternal and maternal control of embryonic growth. Nor can it explain why paternal genes are also imprinted. Thomas (Thomas 1995) theorised that imprinting was a defence mechanism against aneuploidy but the effects of aneuploidy tend to manifest as abortion relatively late in gestation. As mentioned before, an imprinting advantage would be to abort defective offspring before much maternal resources have been utilised.

Thomas also (Thomas 1995) conceived a theory that imprinting could be a surveillance mechanism for chromosome loss. This suggests that if imprinted genes were found throughout the entire genome that they could be used as a sort of early warning system for partial or total chromosome loss or gain. This is because if some or all of a chromosome is lost then the organism will have lost expression of the only expressed allele of a gene instead of one of the pair of expressed alleles in the case of most genes which makes it easier for the cells to detect the loss. This could benefit the organism in two ways, first by protecting the organism from cancer and also by enabling early detection of fetal chromosome imbalances.

A further theory suggested by Varmuza and Mann (Varmuza 1993) is that imprinting protects the mother from trophoblast tumours. Since the trophoblast does not develop well from parthenogenic embryos, the mother is protected from potentially invasive trophoblast-like tumours. However again the theory does not explain paternal silencing. Also angiosperm plants do not have an invasive trophoblast but still have also evolved an imprinting mechanism so the theory is not sufficient in this regard.

If a cell used the total loss of expression of an imprinted gene as an indication of partial or total chromosome loss then cells from the immune system may require the proteins from the imprinted gene to be expressed to allow the cell to live. Without these gene products the cell would fail to pass the immune systems checkpoint and could be eliminated. Alternatively, the imprinted genes could be required for cell division so that when lost the cell is unable to divide and again is eliminated. However, it has been difficult to find evidence to support this theory, as imprinted genes would need to be present on every chromosome and for every one to have a strong deleterious effect if lost.

In summary, it is obvious that each theory does have some merit, but none are sufficient to explain the existence of imprinting in the genome. Currently the most popular theory is the parent offspring conflict hypothesis, which has the most evidence to support it.

1.3: Imprinting disease, growth disorders and cancer.

1.3.1 Disease and growth disorders

The expression of *Igf2* has an important role in the rare overgrowth disorder Beckwith-wiedemann syndrome (BWS) (Wiedemann 1983), (Dell 1997). BWS is a congenital overgrowth syndrome characterised by somatic overgrowth, macroglossia, abdominal wall defects and a susceptibility to childhood tumours especially Wilms tumour of the kidney (Wiedemann 1983) . Both *Igf2* and another imprinted gene called $p57^{kip2}$ are associated with BWS. $p57^{kip2}$ is maternally expressed and codes for a cyclin-dependent kinase (CDK) inhibitor (CKI) (Bourcigaux 2000). Both of these genes are expressed in all the tissues affected by BWS (Lee, Pintar et al. 1990) and it is thought that a loss of imprinting of *Igf2* leading to the activation of the normally silent maternal allele, and the loss of function of $p57^{kip2}$ can cause BWS (Table 1). Loss of function of maternal $p57^{kip2}$ was observed in 5-10% of sporadic (non-familial) cases of BWS that occur (Hatada I. 1996).

IGF2 expression levels were higher than normal in some, but not all patients with BWS (Weksberg 1993). Disruption of *Igf2* imprinting results in mice displaying some of the symptoms of BWS, but not all (DeChiara 1990). Equally over expression of *Igf2* does not demonstrate the full BWS phenotype fully either (Leighton 1995). Chimeras have been made from embryonic stem cells containing an *Igf2* transgene. These animals over-expressed *Igf2* and showed some of the features of BWS during development [Sun F., 1997 #65].

Igf2r is located on mouse chromosome 17 and is also imprinted. It is maternally expressed starting at E6.5. Inheritance of a paternal mutant copy (null allele) of the gene had no phenotype whereas inheritance of a maternal mutant copy resulted in mice with cardiac abnormalities, a tail kink, increased levels of circulating *Igf2* and overgrowth (they were 30% larger than their siblings) (Lau M. 1994).

A paternal deletion of the 15q11-q13 human chromosome occurs in 60-70% of cases of Prader-Willi syndrome (PWS). This disease is characterised by hypogonadism, mental retardation, hypotonia and small hands and feet. This region was found to be imprinted when the disease Angelman syndrome (AS) was found to occur in patients with a maternal deletion of chromosome 15q11-q13. Angelman syndrome is characterised by mental retardation, a puppet-like ataxic gait, hyperactivity, seizures and hypotonia (Lalande

1997) . One or other of these disorders occurs 1 in 15,000 births with familial recurrences. These disorders occur as a result of breakpoints at particular regions of chromosome 15q11-13. There are four genes in this region, which are possible candidates for these disorders, CYFIP1, GCP5, NIPA1 and NIPA2 .

Maternal uniparental disomy of the gene *Grb10* has been observed in 10% of human patients with the disease Silver-Russell syndrome (SRS). This disease results in growth retardation, congenital hemihypertrophy and craniofacial abnormalities. (Wollmann 1995) (Table 1). Regions 7p11.2-p13 and 7q31 have been associated with SRS as well as possible candidates on chromosome 17 .

1.3.2 Cancer

In patients with colorectal cancer hypomethylation of IGF2 DMR1 and the H19 DMR resulting in loss of imprinting (LOI) of this region (Table 1). This hypomethylation is found both in the tumours and in the non-cancerous mucosa of the same patients (Cui 2002). This has been found in 30% of colorectal cancer patients and in only 10% of healthy patients. It has therefore been suggested that testing for LOI in this region can be used as an indication of the risk of developing colorectal cancer (Cui 2003). In mice in which the methylation gene *Dnmt1* had been mutated, reducing the level of methylation in the genome to 10%. These mice later (at around 6months of age) developed T cell lymphomas indicating that DNA methylation can play a role in tumour formation (Gaudet 2003).

Most Wilms' tumours are not associated with BWS but usually exhibit loss of imprinting of *Igf2*. With a large proportion of Wilms' tumours in which loss of imprinting of *Igf2* has been observed, silencing of the normally maternally expressed *H19* gene (codes for an untranslated RNA) has also been identified (Moulton, Crenshaw et al. 1994). The *Igf2* antisense transcript is also over expressed but only in some of the Wilms tumours examined (2 out of six tumours). In any tumours where *Igf2* imprinting was normal, *Igf2* antisense imprinting was also maintained (Vu 2003). In experiments carried out on Wilms's tumour cell lines it was found that the addition of *Igf1* and *Igf2* resulted in apoptosis and necrosis of the tumour cells (Granerus 2001).

The *Igf2r* gene is thought to have tumour supressor effects in humans. It inactivates *Igf2* so loss of *Igf2r* increases cell proliferation and reduces cell apoptosis. LOH of *Igf2r* was observed in 61% of patients with hepatocellular carcinoma (Yamada 1997).

Examinations of human squamous cell carcinomas of the lung had a loss of heterozygosity (LOH) of *Igf2r* in 58% of cases (Kong F. 2000). (Table 1).

Indirect links between imprinting and cancer can be observed in other tumour types. Hydatidiform moles arise from androgenetic embryos and ovarian teratomas arise from parthogenetic embryos. Complete embryogenesis requires both the maternal and paternal genomes.

Region	**Mutation**	**Result**
Igf2	LOI (biallelic expression)	BWS, Colorectal cancer, Wilms tumours,
p57kip2	Loss of Function	BWS
H19	LOI (H19 silenced)	Wilms tumours, Colorectal cancer.
Grb10	mUPD	SRS
Igf2r	Maternal null allele	Overgrowth (30%), cardiac abnormalities, tail kink.
Igf2r	LOH	Squamous cell carcinoma of the lung.
Mash2	Maternal null allele.	Placental failure, embryonic lethal.
Maternal 15q11-q13	Deletion	AS
Paternal 15q11-q13	Deletion	PWS
Maternal/ paternal chromosome imbalance	Parthogenetic/ androgenetic embryos	Ovarian teratomas/ hydatidiform moles.

Table 1. Summary of epigenetic changes in imprinting regions and their resulting disorders.
The region refers to a gene or chromosome that was mutated. The mutation refers to what has been altered on the gene and the result refers to what disorder occurs when the gene is mutated in this way.

1.4 Insulin-like growth factor II gene

Insulin-like growth factor II (IGF2) is a fetal and postnatal growth factor found in mammals. The *Igf2* gene encodes a single polypeptide, which is thought to have autocrine, paracrine and endocrine actions (Efstratiadis 1998). It is found on the distal portion of mouse chromosome seven and human chromosome eleven, region 11p15.5 (131.7Mb) (Tricoli 1984). *Igf2* has eight exons with multiple promoters and also different splice variants of transcripts. The IGF2 peptide is structurally and functionally related to the insulin hormone. IGF2 interacts with the insulin-like growth factor type one receptor (IGF1R), the type two receptor (IGF2R) and also the insulin receptor (INSR) with IGF2R having the highest affinity for IGF2. *Igf2* is oppositely imprinted with respect to *Igf2r* with the paternal allele expressed and the maternal allele silenced at most sites of expression (DeChiara 1991). It is expressed in both mesodermal and endodermal tissues during mouse embryogenesis. Biallelic expression is known to occur in the choroid plexus and the leptomeninges of both embryonic and adult mice. *Igf2* is first expressed in the trophectodermal derivatives immediately after implantation, and then is localised in the extraembryonic and embryonic mesoderm and the foregut lining (Lee, Pintar et al. 1990).

The role of *Igf2* as a growth factor was demonstrated by generating mice that lacked a functional paternal copy of the gene. The mutated mice were only 60% of the size of their wild type littermates (DeChiara 1990). Their placentas were also in proportion to their body weights, which was in keeping with *in situ* hybridisation analysis showing that the components that form the chorioallantoic placenta express *Igf2* (Lee, Pintar et al. 1990).

Mouse *Igf2* (Figure 1.) has four promoters with promoter one (P1) driving expression in the placenta and promoters P2 to P4 both in the embryo and in placenta. Imprinted genes influence growth at the level of cell proliferation and apoptosis in the fetus but they can also influence growth by affecting the structure of the placenta and therefore the flow of nutrients to the fetus (Constancia M. 2002). P1 transcripts are expressed in the labyrinthine trophoblast cells in the placenta and when P1 was deleted this resulted in impaired growth when the deletion was paternally inherited. The mutant placentas were 76% the size of wild-type placentas) (Constancia M. 2002). The resulting pups were at birth 69% of the size of wild type littermates but by the age of three months had caught up with their siblings. P2 transcripts are found in the liver and P3 and P4 transcripts are most abundant in the embryo during the latter stages of embryogenesis. There are three regions

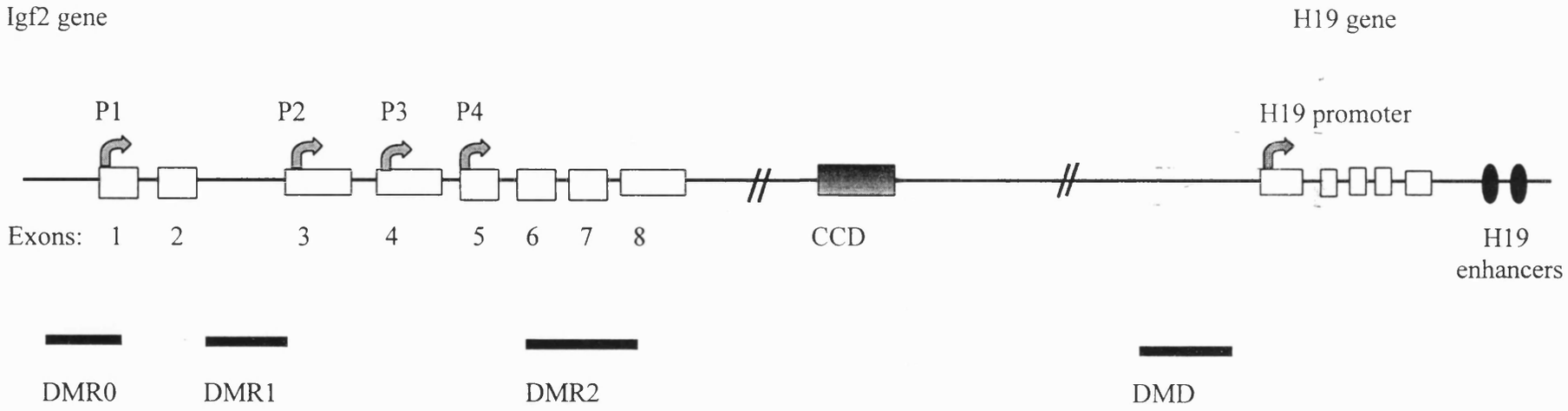


Figure 1. Map of the *Igf2-H19* region of mouse chromosome 7.
P1-P4 refers to the promoters found in *Igf2*. The black lines(not to scale) show the areas of differential methylation (DMR)and the ovals represent the H19 enhancers. The CCD is the centrally conserved domain found between *Igf2* and *H19*.

of differential methylation, one ~3kb upstream from P2 (Differentially Methylated Region One, DMR1), another lies within exons 7 and 8 (DMR2) and DMR0 lies further upstream of *Igf2*. These are discussed in more detail in section 1.7.1.

1.5 H19 gene

The *H19* gene is located about 100kb downstream from *Igf2* (Figure1) and it codes for an untranslated RNA. It may act as a tumour supressor through an unknown mechanism or may act solely to regulate *Igf2* expression. The *H19* gene is oppositely imprinted to *Igf2*, the maternal allele is expressed and the paternal allele is silent. The expression pattern of *H19* is very similar to the expression pattern of *Igf2* except in the choroid plexus and leptomeninges (Ohlsson 1994) . In the stromal cells of the choroid plexus both *H19* and *Igf2* are imprinted, but in the epithelial cells of the choroid plexus there is no expression of *H19* and biallelic expression of *Igf2* (T. Menheniott, personal communication).

Deletion of maternal H19 activated the maternal *Igf2* gene and resulted in mice that were 130% of normal body weight at birth (Leighton 1995). The up regulation of maternal *Igf2* led to the development of the 'enhancer competition model' to explain the link between *Igf2* imprinting and *H19*. The expression patterns and the opposing imprints indicated that the genes may have been competing for the *H19* enhancers (Figure 1). On the unmethylated maternal allele there is nothing to prevent the 3' enhancers up regulating the expression of *H19*. On the methylated paternal allele, the *Igf2* gene competes more efficiently for the *H19* enhancers due to methylation of the *H19* promoter and *Igf2* is therefore the gene expressed on the paternal allele. Experiments carried out on an unmethylated YAC containing the *Igf2/H19* gene cluster showed flaws in the competition model as just described above. It was expected that the YAC would behave as the maternal allele behaved (which is unmethylated in wild type *Igf2*) and that *Igf2* would be silenced (as it is on the maternal allele) and *H19* expressed. What actually resulted was expression from both of the genes (Webber 1998). This model was then further refined following the discovery of a methylation-sensitive boundary in the region between *Igf2* and *H19*, which explains the results observed above (discussed in section 1.7.1.).

1.6 Antisense transcripts

A deletion in the gene *Igf2r* resulted in LOI of the gene and also loss of expression of the antisense transcript. It was shown that this deletion included a promoter for a paternally expressed antisense transcript. When a truncated version of *Igf2r* antisense was maternally transmitted the mice were similar to wild type but a paternally transmitted truncated *Igf2r* antisense gene resulted in mice that were 15% smaller than their wild type littermates. This phenotype can be replicated in mice with biallelic expression of *Igf2r* (Sleutels 2002). When the sense transcript is expressed the antisense is not and when the antisense is expressed the sense is not (Reik 1997). This antisense transcript has been named *Air* and is thought to regulate the imprinting not just of *Igf2r* but also of the neighbouring imprinted genes *Slc22a2* and *Slc22a3* which are also maternally expressed (Sleutels 2002). *Air* overlaps the *Igf2r* promoter and codes for an untranslated RNA. When this was deleted on the paternal allele *Slc22a2* and *Slc22a3* are no longer silenced (Zwart 2001).

Igf2 has three antisense transcripts called *Igf2as-a, Igf2as-b* and *Igf2as-c. Igf2as-b* (Figure 2) is associated with promoter elements and has four exons and so is most likely of the three as transcripts to code for a functional RNA (Moore 1997).

The imprinted gene *Gnas* also has an antisense transcript, which starts in between promoters P1 and P2. *Gnas* is on mouse chromosome 2 and codes for the α subunit of a nucleotide binding protein. Transcripts from the P1 promoter are maternally expressed and transcripts from the P2 promoter are paternally expressed. *Gnas* antisense (*Gnas-as*) is expressed from the paternal allele like the P2 transcript from the paternal sense allele. This is opposite to *Igf2* where the antisense transcript comes from the same paternal allele as the sense transcript. In the case of *Igf2r* and *Gnas* the antisense transcript comes from the allele opposite to the sense transcript (in the case of the P1 promoter transcripts)(Li 2000).

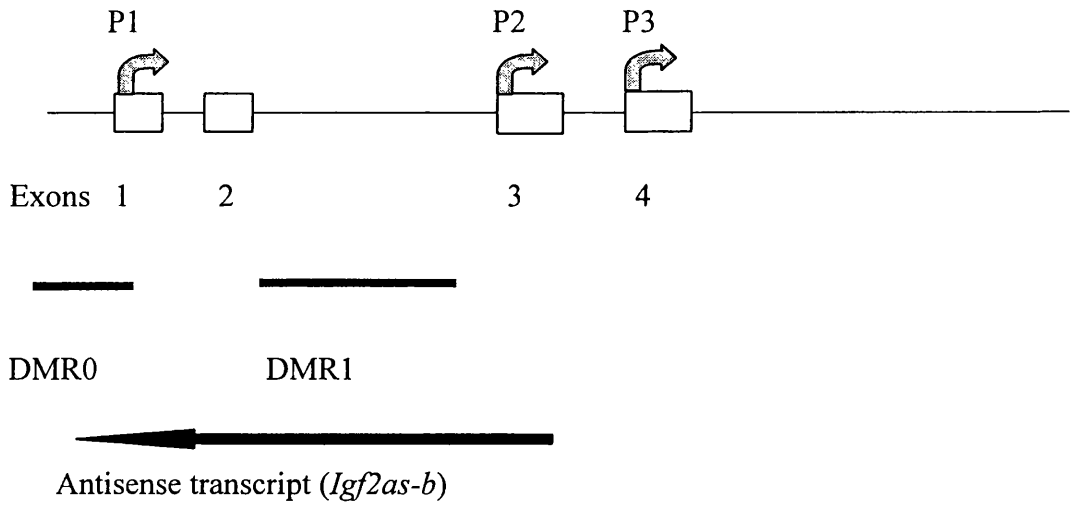


Figure 2.Section of Igf2 gene and all of theIgf2 antisense transcript. (Moore 1997)
P1-P3 refers to some of the promoters found in Igf2. The black lines show two of the three areas of differential methylation. The black arrow shows where the antisense transcript lies and the direction of transcription.

1.7. Epigenetic marks.

1.7.1 Methylation of Igf2 and H19.

Methylation is an epigenetic mark as it does not alter the nucleotide sequence but can be used to distinguish the paternal from the maternal allele. The methylation pattern of *Igf2* is unusual as the expressed paternal allele is the more highly methylated allele. There are three regions of differential methylation associated with *Igf2*.

The first, differentially methylated region 0 (DMR0) is associated with a placenta specific transcript and is located upstream from *Igf2* (Figure 1). This region is maternally hypermethylated in the placenta. In the fetus both the maternal and paternal DMR0s are equally methylated (Moore 1997).

The second region (DMR1) is 600bp long, is located within the 5' portion of the *Igf2* gene (Figure 1) and is paternally hypermethylated (Sasaki, Jones et al. 1992). DMR1 contains a methylation sensitive silencer. Deletion of DMR1 on the maternal allele results in expression of *Igf2* in mesodermal tissues (Constancia 2000). In experiments carried out on DMR1 using bisulphide sequencing a mosaic pattern of methylation was found in a gradient across a CpG island consisting of thirteen CpGs. CpG 1 was found to be most frequently hypermethylated on the paternal allele whereas CpG 13 was found to be paternally hypermethylated with the lowest frequency. There was no CpG that was always

maternally unmethylated and paternally methylated. CpGs 1 to 4 showed the greatest differences having almost twice the frequency of methylation paternally compared to maternally (Feil 1994).

The third DMR, DMR2, is located within the 3' coding region of the *Igf2* gene. DMR2 is paternally hypermethylated in specific tissues such as the foetal liver but is found to be hypomethylated in foetal brain and kidneys. DMR2 contains a methylation sensitive activator (Feil 1994). Deletion of DMR2 on the maternal allele has no effect on *Igf2* expression and a deletion on the paternal allele leads to slightly retarded fetal growth (Murrell 2001).

The *H19* gene located about 100kb downstream of *Igf2* also has a DMR. This DMR (ICR) about 7 to 9kb 5' of the *H19* coding region and is hypermethylated on the inactive paternal allele and hypomethylated on the active maternal allele (Bartolomei, Webber et al. 1993). Deletion of the *H19* DMR results in activation of maternal *Igf2* (Leighton 1995). The *H19* DMR is methylated in sperm but not in oocytes and this is maintained throughout development (except in the germ cells). DMR1 and DMR2 are also methylated in sperm but not in oocytes but this methylation does not remain throughout development so it is thought that H19 DMR is the primary germline modification involved in the regulation of Igf2/H19. Methylation is erased at the morula stage and re-established at post implantation. Deletion of the *H19* DMR on the maternal allele increased methylation levels of DMR1 and DMR2, and deletion on the paternal allele had no effect (Table 2). Deletion of DMR1 on the maternal allele increased methylation levels in DMR2 but had no effect on the *H19* DMR. A paternal deletion did not affect either. Deletion of DMR2 did not effect DMR1 or *H19* DMR methylation. This suggests a hierarchy of DMRs working to establish and maintain the imprint within the *Igf2/H19* gene cluster. When de novo methylation during embryogenesis occurs (*Dnmt*) the already methylated H19 DMR protects maternal DMR1 and DMR2 from methylation, as removal of *H19* DMR results in maternal methylation of these DMRs. Similarly maternally unmethylated DMR1 protects maternal DMR2 from methylation (Lopes 2003).

Deletion	Effect
Paternal *H19* DMR Maternal *H19* DMR	No effect on DMR1 or DMR2. Increased methylation on DMR1 and DMR2
Paternal DMR1 Maternal DMR1	No effect on DMR2 or H19 DMR. Increased methylation on DMR2, no effect on H19 DMR.
Either paternal or maternal DMR2	No effect on DMR1 or H19 DMR.

Table 2. Summary of methylation hierarchy.
This table summarises the effects deletions of Igf2 DMRs and H19DMR has on the methylation of the other DMR's in the Igf2-H19 gene pair (Lopes 2003).

The way in which methylation controls the imprinting of *Igf2* and *H19* involves a DNA binding protein called CTCF. CTCF is a nuclear protein (CCCTC- binding factor) that binds to DNA via a zinc finger (Bell 1999). It can act as a gene activator, repressor, silencer or chromatin insulator by forming complexes with DNA, some of which are methylation sensitive (Ohlsson 1994) . 90kb downstream of the *Igf2* gene lies the imprinting control region (ICR) (also referred to as the differentially methylated domain, DMD). The ICR is methylated on the paternal allele and is unmethylated on the maternal allele. CTCF cannot bind to methylated ICR allowing the *H19* enhancers to upregulate the expression of *Igf2* on the paternal allele. CTCF can bind to unmethylated ICR, insulating *Igf2* from the effect of the *H19* enhancers on the maternal allele (Figure 1) (Li, Beard et al. 1993) (Leighton 1995).

The silencer activity of the ICR can be seen in endodermal tissues where *H19* expression is switched off (Drewell 2000). When a deletion was made in this region and paternally transmitted, silencing was relaxed and the *H19* gene was expressed with loss of *Igf2* expression. Maternal deletion of the 1.2kb region had no effect on *H19* expression. The expression of paternal *H19* was restricted to tissues where the *H19* enhancers have an effect. A second paternal deletion in the ICR led to reactivation of *H19* expression. This second deletion was closer to the *H19* gene showing the silencer activity to lie within the first 0.8kb region of the ICR.

There is also some evidence that CTCF is involved in maintaining differential methylation by blocking the methylation enzymes throughout development. Changes in ICR methylation have also been observed in some human cancers and may explain some cases of *Igf2* overexpression .

In early embryogenesis genome-wide demethylation occurred with methylation levels hitting their lowest point at the blastocyst stage (Monk 1987). This first occurs in the paternal pronucelus and then spreads to housekeeping genes and regions of direct repeats. Imprinted genes escape this demethylation (Santos 2002). Post implantation a genome-wide wave of methylation was shown to occur under the control of the *de novo* methylation genes *Dnmt3a* and *Dnmt3b* (Okano 1999). When the *Dnmt3a* and *Dnmt3b* genes were mutated the *de novo* methylation activity of these genes was lost (Hseih 1999). It is thought that *Dnmt1* is responsible for maintaining methylation on the genome. It is responsible for copying the parental strand methylation imprint onto the daughter strand (Okano 1998). Knocking out *Dnmt1* results in a genome-wide loss of methylation and is embryonic lethal (Li 1992).

The gene *Igf2r* has areas of differential methylation also. This is a maternally expressed gene found on mouse chromosome 17. It has a methylated DMR (DMR2) on the expressed maternal allele (in intron 2) and another DMR (DMR1) on the silent paternal allele (in the promoter region). The maternal DMR originates in the germ cells and is thought to be the original imprint, which differentiates the maternal and paternal alleles (Birger 1999).

The gene pair *Dlk1* and *Gtl2* behaves in a similar way to the *Igf2-H19* gene pair. *Dlk1* (which is compared to *Igf2* here) is paternally expressed and has a DMR which is paternally hypermethylated (as is *Igf2* DMR1) and maternally hypomethylated. *Gtl2* (which is likened to *H19* here) is maternally expressed and has a paternally hypermethylated DMR (Takada 2000). One major difference found between these gene pairs is the location of the CTCF imprinting control centre that lies in intron 1 of *Gtl2* instead of in the inter-genic region (Paulsen 2001).

1.7.2 Acetylation

DNA in chromosomes is packaged into chromatin, which consists of DNA and protein. The proteins the DNA is coiled around are histones, which consist of octamers, made up of two units each of H2A, H2B, H3 and H4. The DNA thread is wrapped around each protein twice (Figure 3.)

During cell mitosis histone acetylation patterns remain and so it is a heritable epigenetic imprint that can influence the expression of genes (Jeppesen 1997). This hyperacetylation is scattered randomly throughout the genome except on the inactive maternally inherited X chromosome where the histones were generally hypoacetylated.

Igf2 DMR1 is rich in acetylated histones with the hypomethylated maternal DMR1 having more acetylation than the paternal hypermethylated DMR1. This could mean that the maternal DMR1 may have a more open chromatin structure than the paternal DMR1. This allows maternal DMR1 to interact with repressors. A protein called MeCP2 that binds to methylated sequences of DNA also forms complexes with histone deacetylase (Bestor 1998). Inhibiting histone deacetylase with trichostatin A activates genes silenced by methylation (Bestor 1998). This indicates that chromatin could be deacetylated through methylation dependent pathways. However mice lacking in MeCP2 do not show biallelic expression of imprinted genes unlike *Dnmt1* knockout mice (Li, Beard et al. 1993).

The *Igf2r* gene has also been examined for histone acetylation. Higher levels were found on the expressed maternal allele than on the silent paternal allele. The antisense transcript was also examined and again where the active allele was found (paternal allele) higher histone acetylation was also found. Treatment of cells with Trichostatin A decreased methylation on both the sense and antisense transcripts (Hu 2000).

Examination of the histone acetylation of *Air* showed that on the expressed alleles, the DMR regions had histone acetylation and the repressed alleles were methylated in these regions. Again treatment with Trichostatin A led to derepression of the silenced alleles resulting in biallelic expression of *Air* (Hu 2000).

The DMR2 of the gene *Snrpn* is associated with PWS as mentioned in Section 1.3.1. Paternal *Snrpn* DMR2 is unmethylated and highly acetylated compared to the maternal methylated DMR.

Methylation ●

Chromatin condensation

Acetylation ■

Open chromatin formation with direction of transcription shown by arrow.

Figure 3. Representation of methylation and acetylation of genome. Based on Figure 2. of Genomic imprinting (Reik 2001).
The cylinders represent the histone protein complexes with the black thread coiled around them representing DNA. Methylation is shown as grey circles and acetylation as grey squares. Transcription enzymes are represented by a white circle.

1.7.3 Replication timing and differences in meiotic recombination.

Fluorescence *in situ* hybridization (FISH) experiments carried out on interphase nuclei revealed a large number of nuclei in which one allele had replicated before the other. Analysis of the alleles of *Igf2* showed differences in replication timing with the paternal allele replicating before the maternal allele . This asynchronous replication is established in the gametes and maintained in the foetus making it an imprinting mark as it also correlates with imprinted expression patterns.

Heterochromatin (tightly coiled chromatin) replicates at a later stage than euchromatin (loosely coiled chromatin, transcribed). These replication-timing differences may have left an epigenetic mark on the chromatin. The accessibility of enzymes to the DNA may have been affected by chromatin structure. High levels of histone acetylation found on active genes correlate with areas of euchromatin (Ouspenski 2003). Usually the paternal allele replicates before the maternal allele (Reik 2001).

Differences in meiotic recombination frequencies are associated with imprinted genes also. The presence of methylation reduces the frequency of meiotic recombination. An inversion on chromosome 7 in the *H19* region was created and called the minute (*Mnt*) mutant. When paternally transmitted *Igf2* is repressed and when maternally transmitted *H19* is silenced. However despite the disruption to the *Igf2/H19* gene region which when maternally transmitted replicates wild type *Igf2/H19* expression, asynchronous replication

is unchanged. This implies that the methylation imprint can be altered without affecting replication timing differences (Cerrato 2003).

However, acetylation and replication timing alone are not sufficient to regulate imprinting patterns in the genome.

2.0 Aims

The general aim of this thesis was to assess the function of DMR1 and the effect this gene region had on the expression of *Igf2*.

This was done in two ways, firstly by deleting DMR1 and crossing these knockout mice with *p53* knockout mice. The significance of *Igf2* in cancer has already been mentioned in Section 1.3.2 and in Section 3.1 the significance of *p53* in cancer is explained. The objective was to ascertain if DMR1 caused p53 knockout mice to develop different tumours than had been observed previously (Donehower 1992), if tumours developed sooner or later than seen before, and if a deletion of DMR1 resulted in heavier or lighter mice than wild type, mice that grew faster or slower than wild type or if any organs were over or underdeveloped. The effects on body proportions of prolonged postnatal *Igf2* expression have not been studied in detail. Previous studies have found that extended postnatal Igf2 expression resulted in a lean phenotype (Rogler 1994), (Ward 1994).

The second way that the effect DMR1 had on *Igf2* expression was examined was by constructing transgenes consisting of elements of the *Igf2/H19* gene region and including reporter genes so as the expression the various constructs had *In vitro* and *In vivo* could be analysed. The constructs were compared to each other to assess the role of each part of the gene region in controlling *Igf2* expression. Male and female transmission of the constructs were compared to ascertain if there were any expression differences due to imprinting effects. Expression between various organs were compared to determine where expression is highest and lowest within the mouse. The effects of methylation were also examined in transgenic mice and in transient transfections to determine if there was any correlation between expression levels observed and methylation patterns detected.

Chapter 2: Materials and methods.

Transient transfections

Transient transfections were carried out on HepG2 cells, which are an endodermal liver cell line. The constructs that were transfected were called M, T, A, E and Me-14 (Figure 1). These constructs have been inserted into a Bluescript vector using restriction sites found in the polycloning site of the vector. M utilised *BamH1* and *EcoR1* restriction sites, T two *EcoR1* sites, A *BamH1* and *Not1* and both E and Me-14 used *EcoR1* and *Not1* sites to insert the construct. The transfections and assays were carried out as described below. Constructs E and Me-14 differ in only one aspect, namely that construct Me-14 has two restriction sites in its DMR (Figure 1) so that the central portion of DMR1 can be excised, methylated and replaced in the construct prior to cell transfection and assay of luciferase levels.

2.1.1.Plasmid preparation (Ward 1997)

LB-agar (per litre: 950ml of deionized H_2O, 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, pH 7.0, 0.2ml NaOH, 15g Bacto-agar, autoclave for 20min at 15lb/sq. in.) with 1μl/ml ampicillian (50mg/ml) was inoculated with scrapings from glycerol stocks stored at –80°C and incubated overnight at 37°C. 2ml of LB-broth (per litre: 950ml of deionized H_2O, 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, 0.2ml of NaOH, autoclave for 20min at 15lb/sq. in.) with 1μl/ml ampicillian (50mg/ml) was inoculated with a single colony from the overnight LB-plate. This was incubated overnight at 37°C in a shaking incubator. 200ml of LB-broth with ampicillian (50mg/ml) was inoculated with 2ml of the overnight culture and incubated at 37°C overnight.

The plasmid extraction was carried out using a 'Qiagen EndoFree™ Plasmid Maxi Kit'. This kit yields ultra pure, endotoxin-free plasmid DNA suitable for transfection of cultured mammalian cells. The DNA was eluted as detailed in the manufacturers handbook. Plasmid purification is based on the interaction between the negatively charged phosphates of the DNA backbone and the positively charged diethylaminoethanol groups that reside on the surface of the resin found in the Qiagen columns.

The plasmid DNA was recovered by centrifugation at 11,000rpm for 10min and resuspended in 200µl TE (10mM Tris.Cl, 1mM EDTA).

2.1.2: Agarose gel electrophoresis and ultra-violet light spectrophotometry. (Sambrook 1989)

A 1% agarose gel (0.5g/50ml) was used (0.5g agarose, 50ml TAE: 0.04M Tris-acetate, 0.001M EDTA). 9µl of plasmid solution with 1µl of loading dye (Promega, 6X) was loaded into each well and electrophoresed at 70Volts for 1hr alongside a 1kb marker (Promega).

The plasmid concentration was estimated in comparison with the 1kb marker and confirmed using UV spectrophotometry. The plasmid was diluted to a 1/100 dilution with TE and the absorbance measured at 260nm. A reading of 1=50µg/ml of plasmid DNA.

2.1.3: Maintenance of HepG2 cells.

HepG2 5ml (per litre: Amphotericin B 250µg/ml (GibcoBrl), 5ml Penicillin/Streptomycin (P/S GibcoBrl), 25ml Fetal Bovine Serum (FBS, GibcoBrl), 500ml DMEM/NUT. Mix.F12 with Glutamax-11 (GibcoBrl)) medium was pre-warmed in a 37^{0}C water bath.

HepG2 cells were removed from frozen nitrogen and thawed. 5ml of HepG2 medium was slowly added to the cells. 25ml of medium was dispensed into a flask and the suspended cells were added to the medium. The medium was changed every 48hrs and the cells were passaged every four days.

2.1.4: Transfection of HepG2 cells.

Two sets of 1.5ml microfuge tubes were labelled, one set for the plasmid DNA and one set for the serum free medium (SFM, GibcoBRL). Into the first set of tubes 2µg of luciferase reporter plasmid construct and 2µg of Beta Galactosidase reporter construct was dispensed.

Into the second set of tubes, 200µl of SFM and 6µl of Fugene 6 (Boehringer) was dispensed. This was left at room temperature for 5min. The SFM/ Fugene 6 mixture was pipetted into the DNA mixture and left at room temperature for 15min. Fresh medium was pipetted into the HepG2 wells in the six-well plates and then 100µl of the above mixture was added to each well. This was gently mixed and left for 48hr at 37^{0}C.

The old medium was aspirated and the HepG2 cells rinsed twice with 10ml of phosphate buffer solution (PBS, per litre: 8g NaCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, 800ml sterile H_2O, pH 7.4, sterilise by autoclaving). 100µl of lysis buffer (25mM Tris.H_3PO_4, 2mM DTT, 2mMCDTA, 10% glycerol, 1% Triton X-100, pH 7.8) was dispensed and left at room temperature for 20min and then transferred into 1.5ml microfuge tubes.

2.1.5 Assay for luciferase.

Solutions of luciferase were prepared from a stock solution at 2ng/1µl to make a standard curve by diluting to 10^{-1}, 10^{-2} up to 10^{-5} ng/µl. 5µl of each cell lysate was loaded in duplicate into 96 well microtitre plate and then the standard curve dilutions were loaded, also in duplicate. Light emission was assayed using a luminometer (EG&G Berthold Microplate Luminometer LB 96V) which dispensed 25µl of luciferase reaction mixture (Promega) and after a delay of 10sec counted emitted light (photons). The resulting figures (relative light units, rlu) were entered into an Excel worksheet, which calculated the log of the rlus and the log of the standard curve rlus. Using the standard curve the total ng of luciferase in the sample was calculated taking into account the dilution factor used in the assay sample well.

2.1.6 Assay for beta-galactosidase.

Beta-galactosidase was co-transfected along with the luciferase construct to compare transfection efficiency. This is a colorimetric reaction where 0-nitrophenyl-β-D-galactopyranoside converts the colourless substrate into a bright yellow liquid as an indicator of beta-gal transfection levels. 20µl of each cell lysate was loaded into another 96 well plate in duplicate and the last two wells were loaded with lysis buffer alone and blanks. 140µl of buffer A (0.06M Na_2HPO_4, pH 7.0, 0.4 M NaH_2PO_4, 0.01M KCL, 0.001M $MgSO_4$, 0.05M β-mercapto-ethanol) 25µl of 0-nitrophenyl-β-D-galactopyranoside (ONPG, 4mg/ml solution of ONPG dissolved in 0.1M sodium phosphate) was added to each well. This was incubated at 37°C for 30min to 2hr and absorbance was measured at 405nm in a colorimeter (Rosys Anthos 2001). The absorbancies were entered into the same Excel worksheet as the luciferase results, but in this case without a standard curve.

2.1.7 Assay for protein

Protein levels were assayed as an indication of relative cell number transfected with the plasmid. This is a colorimetric reaction with a blue colour indicating protein levels in the cell lysis sample. A standard curve was made using BioRad protein assay standard II (BioRad). This was diluted to 1mg/ml. Then the following concentrations of the assay standard were made by dilution using sterile H_2O or 1/10 lysis buffer: 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 using sterile H_2O or 1/10 lysis buffer (as before). Cell lysates were diluted to a 1/10 or 1/20 dilution with sterile H_2O. 10µl of the diluted cell lysates were loaded in duplicate into a 96 well plate together with the standard curve dilutions. 200µl of BioRad assay solutions (BioRad) was added to each well and left at room temperature for 30min. The absorbancies were entered into the same Excel worksheet as mentioned above along with the absorbancies of the standard curve. The worksheet then used the curve to calculate the total amount of protein present in the cell lysis samples again taking into account the dilution factors used.

2.1.8 Data analysis

Three sets of figures were generated in the Excel worksheet, the total amount of luciferase (ng) in the cell lysis samples, the beta-gal absorbancies and the total amount of soluble protein (mg) in the samples. The amount of luciferase was divided by the amount of protein which was then divided by the beta-gal absorbancies to give the total ng of luciferase present in the sample corrected for cell number (protein assay) and transfection efficiency (beta-gal assay). All analysis was carried out using the Prism programme.

2.1.9: Patch methylation transfections.

2.1.9.1: Plasmid preparation, agarose gel electrophoresis and UV spectrophotometery

Construct Me-14 was prepared as described in Section 1.1. of Materials and methods and the plasmid concentration was estimated as described in Section 1.2 of Materials and methods.

2.1.9.2: Patch methylation of construct.

The DNA was split into two tubes and two methylation reactions were set up. One tube was methylated and the other was mock-methylated as a control for the processing steps. The enzyme used to methylate is *Sss*I, which methylates all cytosine's in CpG dinucleotides (NEB). 10 units of methylase were used for every 1-5μg DNA. Into each tube 50μl of DNA was added, 10μl of 10X NEB buffer 2, 10μl *Sss*I (or water), 2μl S-adenosyl-methionine (SAM), 28μl sterile H_2O. These were incubated overnight at 37°C with the SAM renewed after 3-4hr in both tubes.

Then 100ng of the DNA was digested with *Hpa*II for 1-2hr at 37°C. Gel analysis showed that the mock-methylated sample had digested into smaller fragments and the methylated DNA was intact. The DNA was then purified using a Qiagen nucleotide purification kit and eluted in 50μl. The DNA yield was estimated on a gel.

The vector and insert were ligated together at a ratio of 3-5:1 insert: vector with 2μl of ligase used for every 5μg of total DNA. The ligation reactions were incubated overnight at 4°C. The reactions were then purified using a Qiagen nucleotide purification kit and eluted in 50μl as per the manufacturers instructions.

2.2.1 Transgenics

The constructs used in the transgenics experiments all contain DMR1, the P3 promoter and either the luciferase reporter gene or the beta-galactosidase (lacZ) reporter gene. They also contained either or both of the *H19* enhancer and the centrally conserved domain (CCD) (Figure 1) F1 mice (C57BL6/CBA) were used for superovulation as they have a high egg

production rate (between 40 to 60 per mouse). MF1 mice were used as pseudopregnants because they are an outbred line and make good foster mothers.

The mice have a 12hr light/dark diurnal cycle with light occurring between 8am and 8pm. They were assumed to have mated at midnight in the dark cycle of the night before a copulation plug was detected. From this, it was known when embryos had reached embryonic day 14.5 (E14.5) and day 1 post birth (day1).

2.2.1.2 Creating new transgenic lines. (Hogan 1994)

F1 females (as described above) were injected with Pregnant Mares Serum (PMS, mimics follicle-stimulating hormone) 46-48hr prior to ovulation and Human Chorionic Gonadotropin (hCG, mimics luteinizing hormone) 10-13hr prior to ovulation as described in Manipulating the Mouse Embryo (Hogan 1994) to cause superovulation. MF1 females were mated with vasectomised males to generate pseudopregnant mice, which were the recipients of the injected embryos.

The superovulated F1 mice were mated when in oestrus with stud males and the embryos were harvested and suspended in M2 medium (5ml stock A{947mM NaCl, 4.78mM KCL, 11.9mM KH_2PO_4, 11.9mM $MgSO_4$, 232mM sodium lactate, 55.6mM glucose, 0.03g penicillin, 0.025g streptomycin}, 0.8ml stock B {250mM $NaHCO_3$, 0.01% phenol red}, 0.5ml stock C {33mM Na pyruvate}, 0.5ml stock D, {17.1mM $CaCl_2 2H_2O$}, 4.2ml stock E, {250mM HEPES} and 200mg bovine albumin (sigma 96-99%), 39ml sterile H_2O, filter sterilised).

The constructs (prepared as in Section 2.1.2 of materials and methods) were injected into the embryos while they were suspended in KSOM medium (1ml stock A' {950mM NaCl, 25mM KCL, 3.5mM KH_2PO_4, 2.0mM $MgSO_4$, 100mM sodium lactate, 2.0mM glucose, 0.03g penicillin, 0.025g streptomycin}, 1ml stock B {as before}, 0.1ml stock C' {20mM Na pyruvate}, 0.1ml stock D {as before}, 1µl stock F {100mM $Na_2EDTA2H_2O$}, 50µl stock G, {200mM glutamine}, 10mg Bovine albumin (Sigma 96-99%) and 7.8ml sterile H_2O, filter sterilised) using Clark GC100+0 borosilicate glass capillaries (1mm OD x 0.58mm ID, standard wall, with filament) pulled into a long, sharp, tapered needle using a Sutter P-97 Flaming/brown Micropipette Puller using the parameters set in programme 33 (Heat = 275, Pull=170, Velocity = 175, Time = 150). The embryos were held in position using Clark GC100+0 borosilicate glass capillaries (1mm OD x 0.58mm ID, standard wall,

without filament) were polished using Beaudoin Microforge No. 620. A Nikon Eclipse inverted microscope was used to carry out the injections with Nikon Narishige motorised joysticks used to manipulate the embryos.

These injected embryos were transferred into the oviducts of the pseudopregnant mice and the litter was tested for transgenic pups when born by PCR (Section 2.1.3) and Southern blotting (Section 2.1.3).

2.2.2 Preparation of DNA for transgenesis microinjection.

Insert DNA was cut out from the plasmid (at least 50μg) using appropriate restriction enzymes. This was run on an agarose gel using a preparative comb at 60Volts to completely separate vector from insert. Insert was cut out of the gel and purified using a QIAquick Gel Extraction Kit (Qiagen) as per the manufactures handbook.

A Sartorius micro collodion bag (cat no. SM13202K) was placed in a beaker of sterile H_2O for one hour to rinse away the ethanol it was stored in. The volume of the eluted DNA was recorded and transferred into the bag, which was placed in a beaker half full of dialysis buffer (5-10mM Tris pH 7.4, 0.1-0.25mM EDTA in sterile H_2O, filter sterilised). This was left at 4^0C overnight. The dialysis buffer was changed morning and night for the next 48hr.

The dialysed DNA was removed from the bag, its volume recorded and placed in a Costar Spin-X Filter. This was centrifuged at full speed for one min. The concentration of this was calculated using the equation: spec reading x 100 x 50 = Xμg/ml at 260nm and a concentration of 0.5-1ng/μl was used for injection. This was checked on a 1% agarose gel also.

2.2.3 Genotyping of litters.

Ear and tail biopsy samples were obtained when the pups reached three weeks. The ear samples were used for PCR and the tails were used for Southern blotting.

Polymerase Chain Reaction (PCR).

600µl of 50mM NaOH was added to each ear sample and boiled for 10min. This was left to cool and then vortexed for 30sec. 50µl of Tris (pH 8.0, 1M) was added and the samples were vortexed again.

Into labelled PCR tubes 23µl of Reddy Mix PCR Master Mix (Abgene, 1X conc.) was added, 1µl of Primer Mix (from 15µM stocks, 100µl of each primer added) and 1µl of the ear prep mixture. A control was made using just primer mix and Reddy Mix. The PCR was carried out in a Techne Touchgene (Jencon) PCR machine under the conditions set out in programme Geno 60 (30 cycles, 1min 94°C, 1min 60°C, 1min 72°C).

Southern blotting.

About 24µl (or 10µg) of each sample was digested with a suitable restriction enzyme and electrophoresed on a 1% agarose gel, transferred onto Hybond membrane (Amersham) and hybridised with DMR1 probe using the Church and Gilbert method. A DMR1 fragment used as a probe was cut from a construct p17a14 using *Bam*H1. This gave a 2.5kb band which was then purified using QIAquick Gel extraction kit (Qiagen) following the manufacturers instructions. The purified DMR1 fragment was then labelled using ^{32}P-dCTP (Amersham) (see below).

The gel with the digested DNA samples was depurinated for 15min using depurinating solution (0.2M HCL) and then rinsed with milliRO H_2O. It was then immersed in denaturing solution (0.5M NaOH, 1M NaCl) for 15 min. This was then washed twice for 20min in neutralising solution (1.5M NaCl, 50mM Tris-HCL, pH7.2, 1mM EDTA). A blotting tank was set up with 20X SSC (3M NaCl, 0.3M Sodium Citrate, pH 7.0) and the gel was left to blot overnight.

2X SSC was used to wash the membrane, which was then crosslinked on the transilluminator for 2min. Church buffer (per litre: 15ml PB, 60µl 0.5M EDTA, 2.1g SDS, 0.3g Bovine Serum Albumin, 14ml MilliQ water) was warmed to 65°C and 400µl of salmon sperm DNA (100mg.ml denatured and sheared) was boiled for 5min. The membrane, the warmed Church buffer and the salmon sperm DNA were added to the hybridisation bottle and left to hybridise at 65°C for 2hr in a Hybaid hybridisation oven in which samples can be mixed by constant rotation.

To label the probe 20ng of probe DNA was made up to 12.5µl in MQ H_2O and was boiled for 5min and then chilled on ice for 5min. 4µl of 'HiPrime' and 2.5µl of[^{32} P] dCTP to label to a high specific activity was added. This mixture was left at 37^{0}C for at least an hour. Meanwhile a spin column was prepared by plugging the end of a 1ml syringe with glass wool, this was filled with Sephadex G50 and equilibrated by spinning for 4min at 1200rpm. The labelling reaction was made up to 100µl with TE. The labelled probe was added to the spin column and centrifuged at 1200rpm for 4.5min, having placed a 1.5ml-microfuge tube in the tube to collect the flow-through. 100µl of TE was added and it was recentrifuged. 400µl of salmon sperm DNA was added, the mixture reboiled for 5min and chilled on ice for 5min, then added to the prehybridising solution and left rotating at 65^{0}C overnight.

Wash solution (40mM PB, 10gSDS, 2ml 0.5M EDTA) was warmed to 65^{0}C, the hybridising solution was poured off and the tube filled with wash solution for 15min. This was repeated with another 15min wash, followed by a final 1hr wash. The membrane was then rinsed in 480ml MQ H_2O with 20ml PB to remove SDS. Saran wrap was used to wrap the membrane and it was put in a cartridge with film (Biomax-MR, Kodak, X-ray film for autoradiography) and left at –80^{0}C to develop. When it was ready, it was developed using an X-Ograph Compact X2 developer.

2.2.4 Luciferase assays in transgenic tissues

The tails of the Day1 pups and the heads of the E14.5 embryos (heads were homogenised using an Ultra-turrax T8 IKA Labortechnik for approx. 10 sec) were used in a luciferase assay to test which individuals were transgenic. The heads and tails were vortexed in lysis buffer (25mM Tris-phosphate, pH 7.8, 2mM DTT, 2mM 1 2-diaminocyclohexane-N,N,N', N'- tetraacetic acid, 10% glycerol, 1% Triton X-100) and 10µl aliquots were assayed for luciferase in a luminometer (EG&G Berthold microplate Luminometer LB 96V) which dispensed 25µl of luciferase assay substrate (Promega) and after a delay of 10sec counted light emissions (as in Section 1.1.5 of Materials and methods).

A reading of >200rlu was considered positive for the luciferase transgene as a blank control typically gives a reading of 85-120rlu. Any individuals with this result were further assayed. The brain, tongue, liver, kidney and muscle of the Day1 pups and the body, placenta and yolk sac of the E14.5 embryos were assayed as described above.

2.2.5 Protein assays in transgenic tissues.

A protein assay was carried out on the E14.5 heads and on the brain, tongue, liver, kidney and muscle of the Day1 transgene positive pups and the body, placenta and yolk sac of the E14.5 transgene positive embryos. A standard curve was made as described before (Section 1.1.7 of Materials and methods). The samples were diluted with water as follows: Brain 1/50, tongue 1/20, liver 1/50, kidney 1/20 and muscle 1/10. Head 1/20, body 1/50, placenta 1/50 and yolk sac 1/20. 10µl of these diluted samples was dispensed in duplicate into a 96 well plate and the absorbencies measured at 405nm in a colorimeter (Rosys Anthos 2002).

2.2.6 LacZ staining of tissues.

Tissue stain base solution (0.1M PO_4 buffer{ 40.5:9.5 of Na_2H:NaH_2, pH 7.4}, 2nM $MgCl_2$, 0.015 sodium deoxycholate, 0.02% Nonidet P-40, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$) was warmed to 37°C and kept in the dark. A 1/40 dilution was made with the X-Gal stock solution (40mg/ml X-Gal dissolved in DMSO) using the warmed tissue base-stain solution and kept in the dark at 37°C until needed.

The tissues were fixed in Tissue fixative (0.1M PO_4 Buffer, pH7.4, 4% paraformaldehyde, 2mM $MgSO_4$, 5mM EGTA) for 45min to 1.5hr on wet ice. Then they were rinsed in Tissue rinse solution A (0.1M PO_4 buffer, 2mM $MgCl_2$, 5mM EGTA) and washed in Tissue rinse solution A for 30min at room temperature. The tissues were rinsed in Tissue rinse solution B (0.1M PO_4 buffer, 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40) and washed in Tissue rinse solution B for 5min at room temperature. The tissues were drained and the complete β-Gal Tissue stain solution was added and left at 37°C in the dark for >1hr.

2.2.7 DNA extraction from mouse tissues.

Approx. 500mg of each organ was placed in a labelled tube and 525µl of tail buffer (50mM Tris, pH 8.0, 100mM EDTA, 100mM NaCl, 1% SDS , 265ml sterile H_2O) and 35µl 10mg/ml protinase K was added. This was left at 55°C overnight.

2µl 0.2mg/ml RNase A was added and the sample incubated at 37°C for 1hr. 200µl of 5M NaCl and 700µl of chloroform:isoamylalcohol (24:1) was added and left mixing on

a rotator for 2hr. These were centrifuged at 10,000rpm for 10min and the upper aqueous layer was dispensed into a fresh tube. This was centrifuged at 15,000rpm for 15min and the supernatant was removed. The remaining pellet was then washed for 1hr at 4°C with 300µl of 70% ethanol. The ethanol was thoroughly removed and the pellet resuspended in 200µl of TE (10mM Tris, pH 8.0, 1mM EDTA and sterile H_2O) overnight at 4°C.

2.2.8 Southern blotting of mouse tissues.

The tissues were digested with restriction enzymes and blotted as described in Section 2.2.1.3 of materials and methods.

2.3. ΔU2/ P53 Mouse Experiments.

Mice aged >9 months were culled and blood samples and selected organs removed. The organs preserved were the brain, a liver section, spleen, pancreas, gonadal fat pads, renal fat pads and the left hind leg.

The mice were culled and a syringe was used to extract aortic blood from the aorta of the heart (~1ml). This was then left at 37°C for 1hr, and then centrifuged at full speed for 5min, the serum removed and stored at –80°C. The samples were then sent for analysis to Dr. Domenico Accili ay Columbia University.

The organs were preserved in PFA (4% paraformaldehyde in PBS, 5 PBS tablets in 500ml H_2O, 20g paraformaldehyde left on heated stirrer until it reached 60°C. Added drops of NaOH until PFA dissolved pH 7) overnight at 4°C, and then embedded in wax for histological analysis. The organs were stained with eosin and heamotoxilin (E+H)and the livers were also stained with PAS to detect glycogen.

Any tumours removed were also preserved as above and stained with H+ E also and sent for pathological analysis to Sweden.

Ear and tail biopsies were taken for genotyping as described in Section 2.2.3 of Materials and Methods.

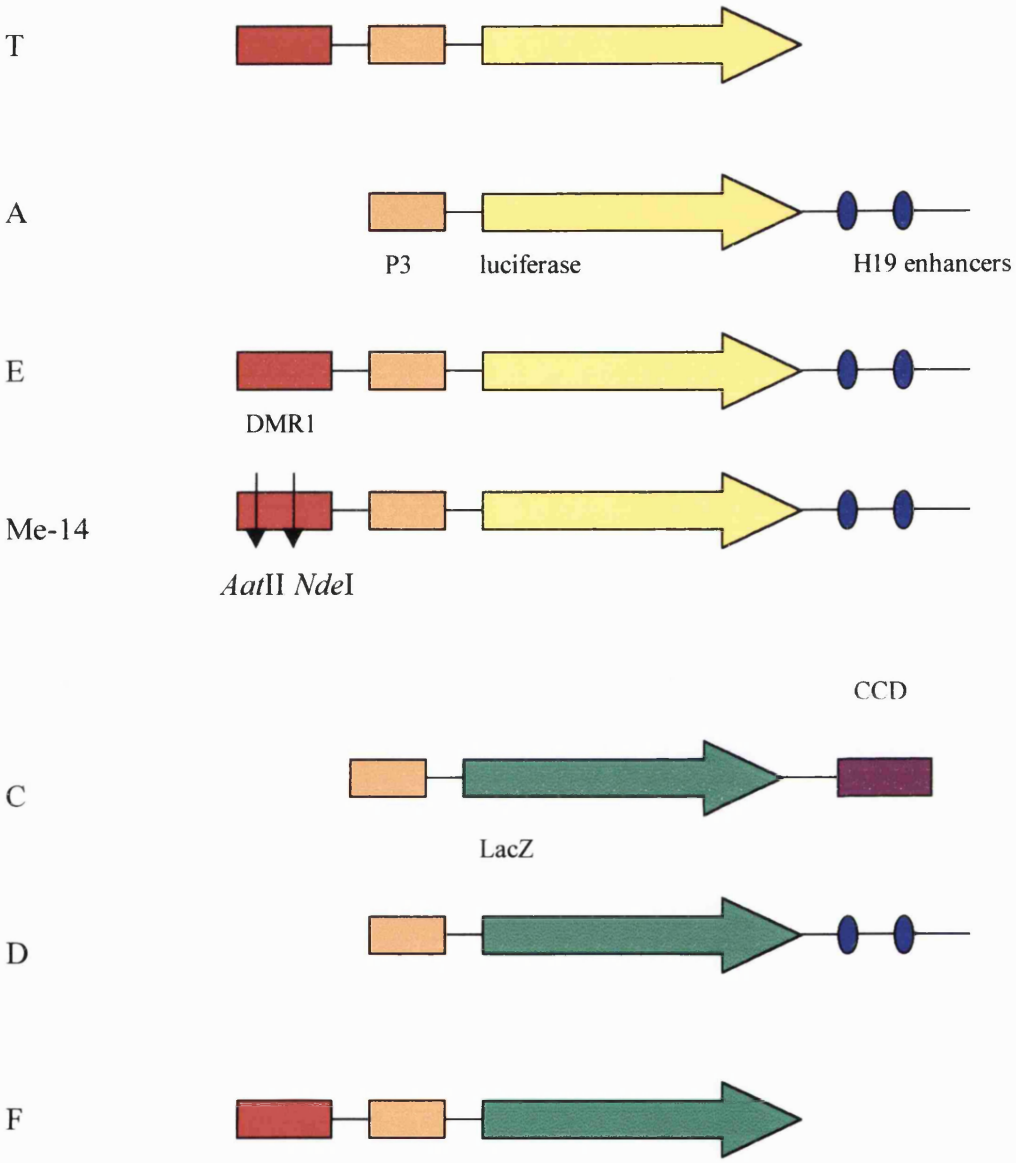


Figure 1: Constructs used in transgenic mice and transient transfections.
Letters denote construct name. The regions represented are: Differentially methylated region 1 (DMR1 red boxes), the *Igf*2 P3 promoter (orange boxes), luciferase reporter gene (yellow arrow), lacZ reporter gene (green arrow), H19 enhancers (blue circles) and the centrally conserved domain (CCD, purple boxes. Black vertical arrows denote the restriction sites *Aat*II and *Nde*I.

Chapter Three: Analysis of growth and tumour formation in mice doubly mutated for p53 and Igf2ΔU2 knockout alleles

3.1 Introduction:

p53 knockout mice (Donehower 1992)

The *p53* gene restrains mammalian cells from entering the S phase halting it at G1. Mammals with a mutated from of the gene have a predisposition to various cancers. *P53* signals to a cell when it has been damaged. The cell death associated protein kinase DAPK sends stress signals to $p14^{ARF}$, which controls a protein called MDM2. MDM2 causes the destruction of *p53* in normal cells, but in cancer cells there is a loss of $p14^{ARF}$ leading to overexpression of MDM2 and inactivation of *p53* (Jones 2001). Mutations in *p53* have been observed in more than 50% of all spontaneous human cancers. Li-Fraumeni syndrome is also associated with *p53*. Usually cells with one normal copy of the gene do not form tumours and cells in which both copies have been altered frequently go on to develop into tumours.

The *p53* gene is dispensible for normal development and *p53* protein levels are normally very low in wild-type cells. Human deletions of *p53* are associated with tumours of the liver, bladder, brain, lung, breast, colon, oesophagus and ovary (note the prevalence in female reproductive organs) (Donehower 1992). However, mice in which both alleles of *p53* have been inactivated by gene targeting develop tumours at a very early age, usually before 6 months. The tumour spectrum observed in *p53* null mice included lymphomas, mostly in the thymus and some in the heart, lung, spleen, liver, kidney and brain, and sarcomas, mostly haemangio-sarcomas. When *p53* wild type, heterozygous and null mice were exposed to 1,2-dimethylhydrazine (DMH) over a period of 16 weeks it was found that the null mice went on to develop carcinomas of the colon but the heterozygotes and wild types did not. When the experiment was carried out for a longer period of time however the significant difference in the occurrence of tumours disappeared. This implies that *p53* null mice may have accelerated tendencies towards tumour formation initially, but this effect is lost over time (Sakai 2003). Investigations carried out on T cells in *p53* null mice revealed that the cells were predisposed to become polyploid more frequently than in wild type mice. This is due to a failure at the mitotic checkpoint during cell division. *P53* null mice spontaneously develop tumours of the spleen and thymus as do *p53* heterozygous mice but at a lower frequency. (Baek 2003).

Exons 2 to six of the gene were deleted in the mice used for this experiment (Clarke 1993). Unlike previous *p53* knockout mice these mice developed tumours in the gastrointestinal system as well as in the thymus, spleen, heart, lungs, liver, kidneys and brain. These mice were shown to have a *p53* dependent pathway to apoptosis in thymocytes.

Igf2 ΔU2 mutant mice (Constancia 2000)

A 5kb region at the 5' end of *Igf2,* including differentially methylated region one (DMR1) and the adjacent repeat were deleted in mice and the effect of the deletion was examined following both maternal and paternal transmission. It was thought that DMR1 contained a silencer that was functional in the unmethylated maternal allele and that deletion of this silencer would instigate expression of Igf2 from the maternal allele. Following maternal transmission of the *Igf2*ΔU2 allele tissues of predominantly mesodermal origin were found to have biallelic expression of *Igf2* (heart, kidney and lung), and endodermal (liver) and placental tissues retained paternal expression of the gene. Maternal transmission of the deletion also resulted in continued postnatal expression of *Igf2* from the maternal allele. Expression from the paternal allele was similarily prolonged following paternal transmission by postnatal day 10. Paternal transmission otherwise resulted in a transcription rate the same as in wild type mice, but with intrauterine growth retardation associated with lack of expression of placenta specific transcripts. These mice were at birth 71% the weight of normal mice but catch up to their wild-type littermates after weaning. The deletion of DMR1 and the adjacent repeat had no effect on *H19* expression.

3.2 Aims:

As part of this study *Igf2*ΔU2 and *p53* knock out mice were crossed with each other, these mice were weighed once a week and if a weight loss of more than 10% of the total body weight of the mouse was detected the mouse was culled and dissected with the aim of detecting tumours. Also any tumours detected independently of a 10% weight loss were similarly culled and processed for histology. This was done to determine if the tumour spectrum changed when *p53* knockout mice also had a deletion in DMR1 and also to see if any tumours developed sooner or later than expected. The postnatal effects of prolonged *Igf2* expression associated with the *Igf2*DMR1 mutant have not been studied in detail. Here we have looked for changes in body proportions and have included analysis of white adipose tissue since previously, prolonged expression of IGF2 transgene has resulted in a

lean phenotype (Rogler 1994; Ward 1994). Any remaining mice were dissected and their organs were weighed and their blood analysed for circulating glucose levels to determine if any organs were disproportionally larger or smaller and whether there was any correlation between glucose levels and body fat.

3.3 Results:

In total 96 mice were weighed over a period of 12 months.

82 of these mice were culled and 14 died of unknown causes .Of the 82 that were culled 10 were because of a greater than 10% weight loss , 9 because of an obvious tumour , 44 were culled to analyse their brain, liver, kidney and gonadal fat pad weights and 19 were culled to analyse their tissues at the end of the experiment.

3.3.1 Mice that died of unknown causes

The 14 mice in this group that died of unknown causes were spread over six genotypes as shown in Table 1. This is also represented graphically in Figure 1.Most of these were p53het (8 mice), 5 were p53nulls and only one was a p53 wild type. 4 had a maternally inherited deletion of DMR1, 4 had a paternal deletion of DMR1 and 6 were wild type for DMR1. No single genotype was significantly represented in this group of mice.

Genotype	Number of mice in sample	No. of mice that died of unknown causes.
ΔU2hetM/p53het	29	3
ΔU2hetM/p53null	9	0
ΔU2hetM/p53wt	7	1
ΔU2hetP/p53het	9	1
ΔU2hetP/p53null	5	3
ΔU2hetP/p53wt	2	0
ΔU2wt/p53het	14	4
ΔU2wt/p53null	20	2
ΔU2wt/p53wt	1	0
Total	**96**	**14**

Table 1. Summary of mice that died of unknown causes.
ΔU2 refers to the DMR1 status of the mice, hetM is a maternal deletion of DMR1, hetP is a paternal deletion of DMR1 and wt is wild type DMR1. p53het mice are heterozygous for the p53gene, p53nulls are knockout mice and p53wt are mice that are wild type for the p53 gene.

Figure 1.

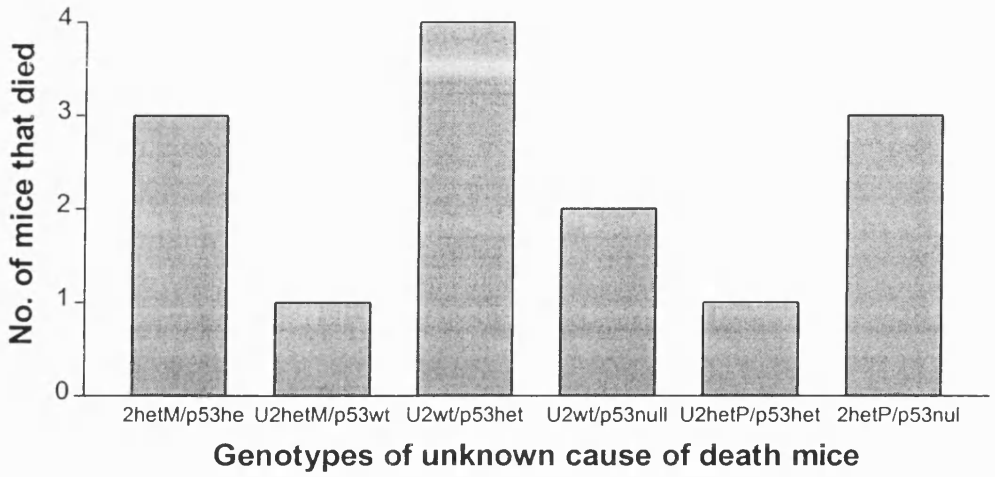


Figure 1. Genotypes of mice that died of unknown causes.
U2 stands for deletion of DMR1, M or P stands for a maternal or paternal deletion of DMR1.p53wt are mice that are wild type for the p53 gene, p53het are mice that were heterozygous for the p53 gene and p53null are p53 knockout mice.

The ages at which these mice that died of unknown causes ranges from 3 months to 19 months with the majority of mice dying after 12 months of age (Figure 2). This is not significantly different from what was observed with the p53 knock out mice and so the loss of DMR1 did not cause this group to live for a longer or shorter period. There is no statistically significant difference between p53het mice and p53 null mice ($p= 0.153$) and there were insufficient samples to compare p53het with p53null. There were also no differences in the age at death between the maternally deleted DMR1 and DMR1 wild type ($p= 0.114$). Paternally deleted DMR1 was also not any different to wild type DMR1 ($p= 0.261$) and there were no differences between maternal and paternal deletions of DMR1 ($p= 0.057$).

Figure 2.

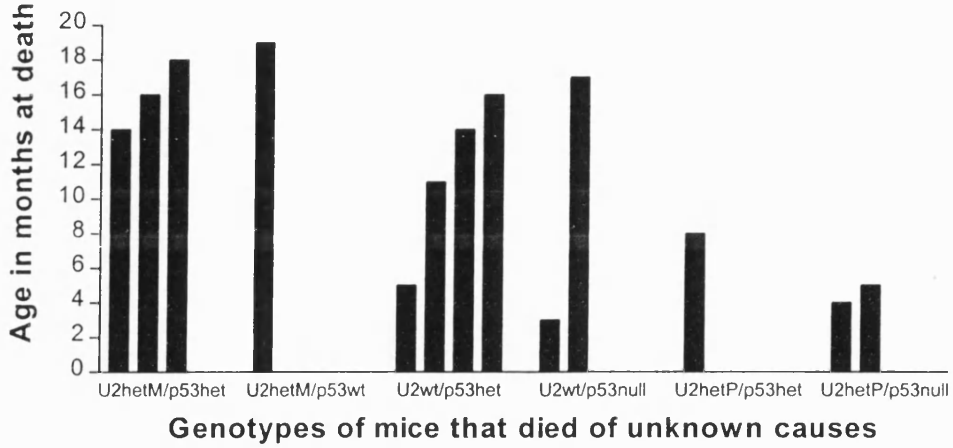


Figure 2. Genotypes of mice that died of unknown causes and the ages at which they died.
U2 stands for deletion of DMR1, M or P stands for a maternal or paternal deletion of DMR1.p53wt are mice that are wild type for the p53 gene, p53het are mice that were heterozygous for the p53 gene and p53null are p53 knockout mice.

The 82 remaining mice fell into four different categories. Firstly there were those mice that lost greater than 10% of their total body weight in one week, secondly there were those mice that developed obvious tumours, thirdly were the mice that were culled in order to weigh their organs and analyse their blood and finally the remaining mice were culled at the end of the experiment and their tissues examined (Figure 3.).

Figure 3.

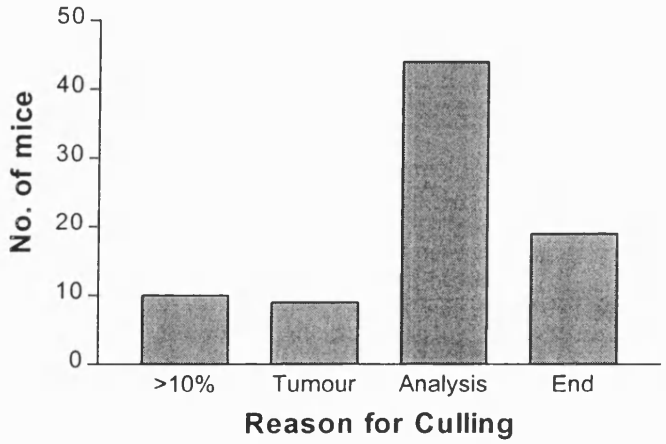


Figure 2.Reason for culling of mice that did not die from unknown causes.
This bar chart represents the reasons the 82 mice were culled.
10 were culled due to a greater than 10% loss in total body weight (>10%), 9 were culled due to the appearance of an obvious tumour (tumour), 44 were culled to analyse their aortic blood and organ weights (analysis) and 19 were culled and their tissues examined at the end of the experiment (end).

3.3.2 Mice that lost more than 10% of their total body weight in one week

Weight loss of 10% or more was used as an indication of possible tumour formation before a visible tumour could be detected. 10 mice out of the 96 lost >10% of their total body weight in one week and 8 of these 10 had an organ abnormality (Figure 4.). 2 mice had no visible cause for the weight loss. The majority of these mice had developed tumours of the thymus and one had an enlarged spleen. Thymus tumours are not unusual in p53 knock out mice and an enlarged spleen can be an indication of an infection rather than any genetic cause for the altered size of the spleen.

Figure 4.

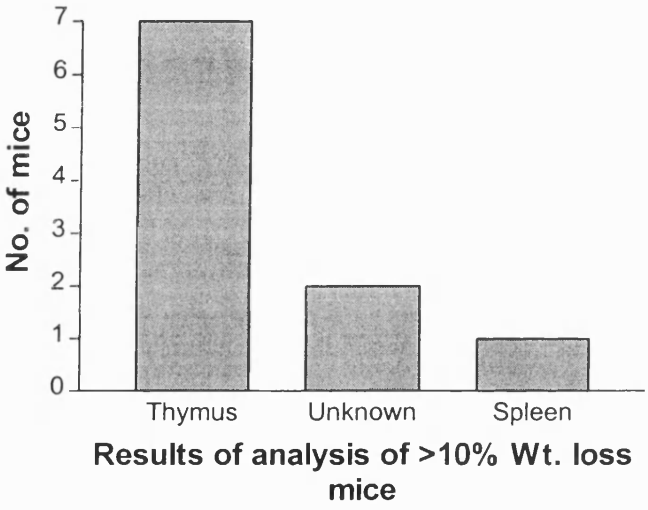


Figure 4. Cause of death of mice that lost >10% in one week.
This bar chart represents 10 of the 82 culled mice. These 10 mice had a greater than 10% weight loss from one week to the next. When dissected 7 were found to have thymus tumours (Thymus), 2 had lost weight due to unknown causes (Unknown) and one had an enlarged spleen (Spleen).

The majority genotype observed in the mice that lost >10% total body weight in one week was the ΔU2wt/p53null genotype (Figure 5.). It accounted for half of the mice in this group. When added to the 2 mice that were also null for *p53* but had a paternal deletion of DMR1 it is now 7 out of the 10 mice that were in this group.

Figure 5.

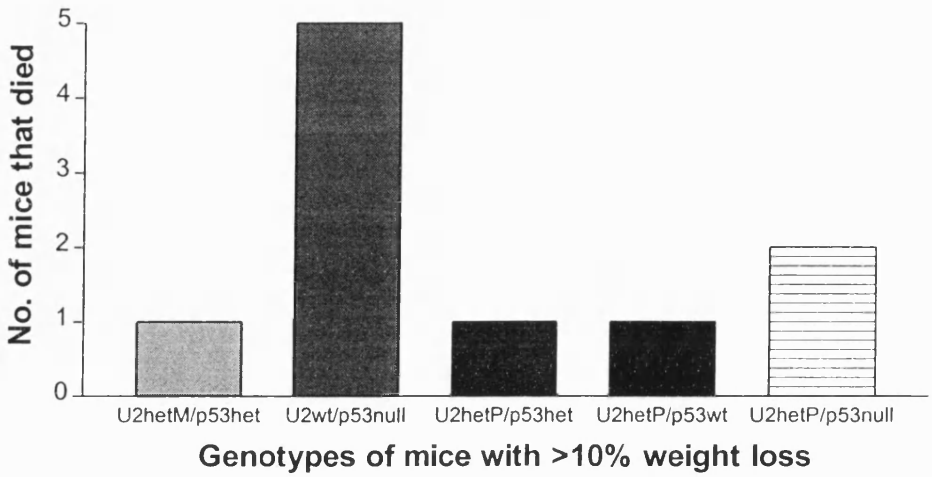


Figure 5. Genotypes of mice that lost more than 10% of their total body weight in one week.
U2 stands for deletion of DMR1, M or P stands for a maternal or paternal deletion of DMR1.p53wt are mice that are wild type for the p53 gene, p53het are mice that were heterozygous for the p53 gene and p53null are p53 knockout mice.

The *p53*null mice were the mice that had tumours of the thymus most frequently which correlates with previous findings of *p53* knockout mice developing tumours of the thymus (Figure 6.). The mice that had an unknown reason for the weight loss were *p53* wild types and had either a maternal or paternal deletion of DMR1. It is possible that their weight loss was not due to tumour formation but some other pathological factor. The mouse with the enlarged spleen was also a *p53* null but wild type for DMR1 and may have had an infection, which led to its weight loss.

Figure 6.

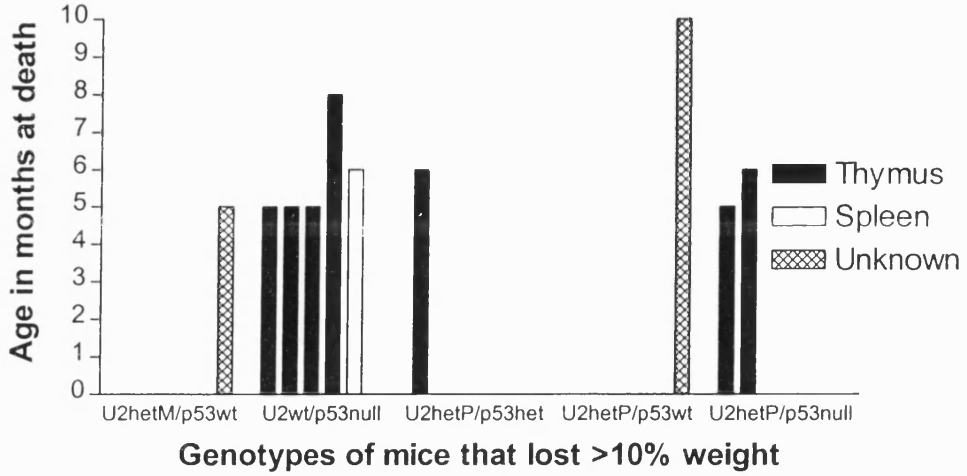
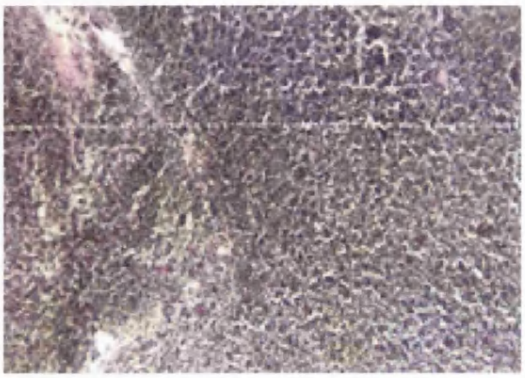
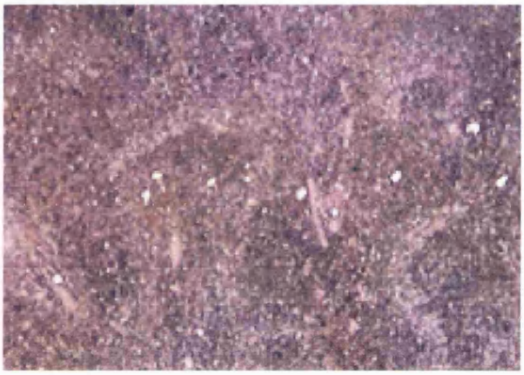


Figure 6. Genotypes of mice that lost more than 10% of their total body weight in one week and the ages at which they were culled.
U2 stands for deletion of DMR1, M or P stands for a maternal or paternal deletion of DMR1.p53wt are mice that are wild type for the p53 gene, p53het are mice that were heterozygous for the p53 gene and p53null are p53 knockout mice. Thymus represents thymus tumours, spleen represents and enlarged spleen and unknown represents no known cause for the weight loss in these mice.

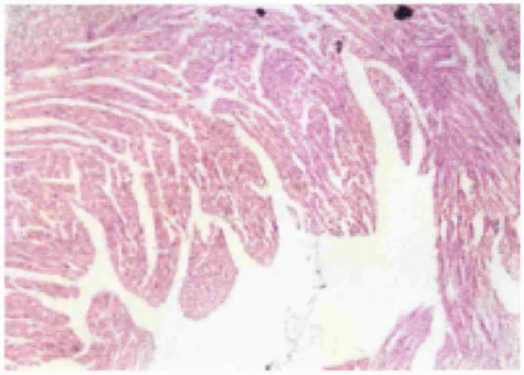
Thymus tumour (1), thymus tumour (5) and enlarged spleen (19) were sent to a pathologist for analysis. The diagnosis given for both of the thymus tumours were malignant lymphomas and for the enlarged spleen as simply a spleen with enlarged structures (Figure 7).



Thymus tumour (1)



Spleen (19)



Thymus tumour (5)

Figure 7. Tissue sections from mice with >10% weight loss.
The sections were stained with H+E and are shown here at x20 magnification. The numbers in brackets refer to the numbers given to tumours and altered organs for identification.(see table 2 for details)

3.3.3 Tumour development

Of the 9 mice that developed abnormalities without any detectable weight loss the majority had abnormal lymph nodes (3 out of 9 mice). Most of the abnormalities were found in the mice with a maternal deletion of DMR1 and were heterozygous for p53 (Figure 8.). None of the mice that developed abnormalities were wild type for *p53* or DMR1. The tumour spectrum in this group is more varied than in previous groups but is not statistically different from previous findings.

Figure 8.

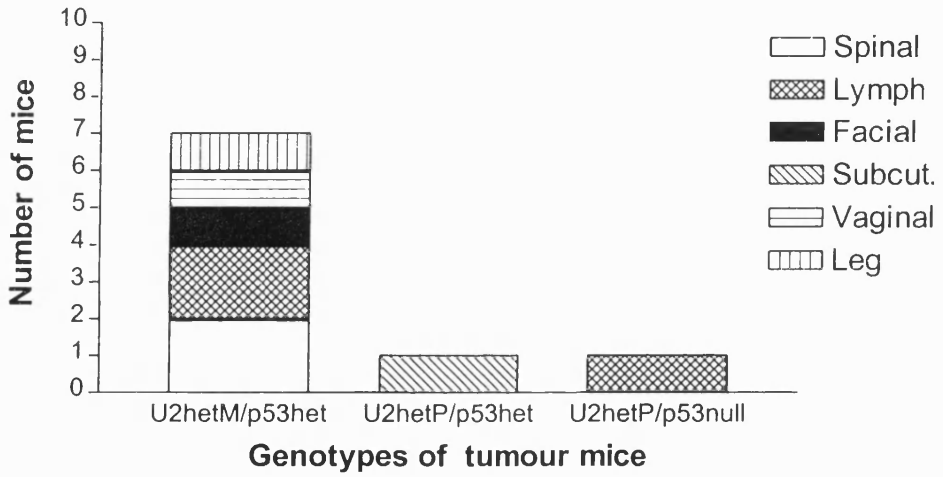


Figure 8. Genotypes and tumours of mice that developed tumours without any weight loss.
U2 stands for deletion of DMR1, M or P stands for a maternal or paternal deletion of DMR1.p53wt are mice that are wild type for the p53 gene, p53het are mice that were heterozygous for the p53 gene and p53null are p53 knockout mice.1 subcutaneous tumour (Sub), 2 spinal tumours (Spinal), 1 vaginal (Vag), 3 lymph node tumours (Lymph), 1 facial tumour (Facial) and 1 mouse with a leg abnormality (leg).

There is no statistical difference between the three genotypes regarding the ages of the mice at death (Figure 9.). However when compared to the group of mice that showed weight loss before physical abnormalities there is a significant difference between the two groups ($p= 0.0039$). The mice that showed >10% weight loss died sooner than the mice that developed abnormalities. Also the >10%weight loss group mostly developed thymus tumours whereas the second group mostly developed lymphomas. It appears that thymus tumours can be associated with weight loss and lymphomas are not. The weight loss could be due to changes in metabolism due to alterations within the thymus as the thymus not

only controls immune mechanisms but has been associated with eczema, lupus, some forms of diabetes, anaemia, hypoglycaemia and renal failure.

Figure 9.

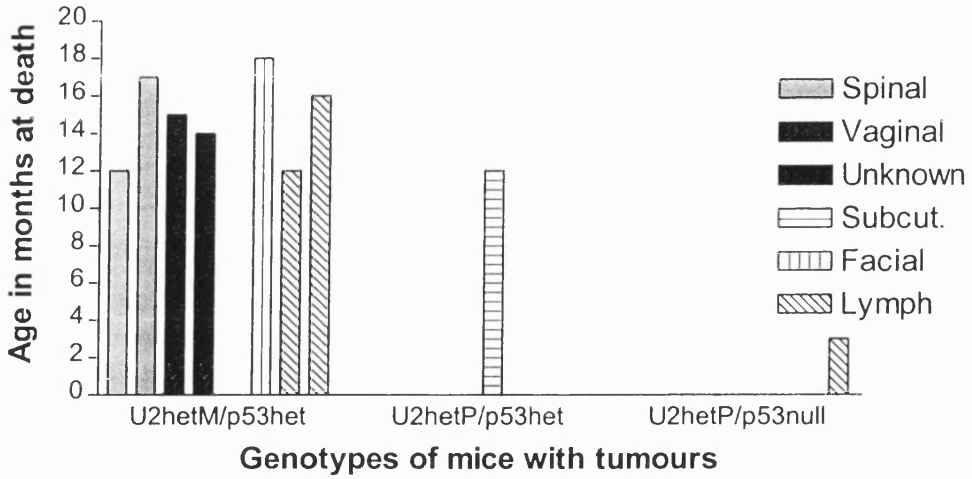
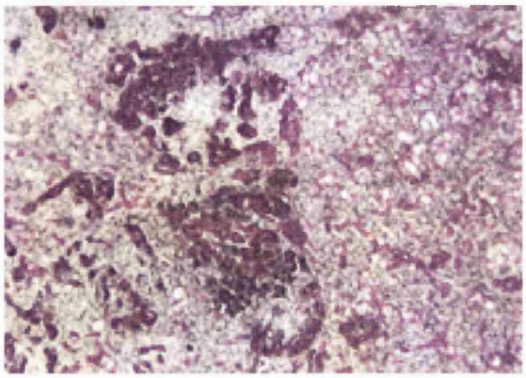


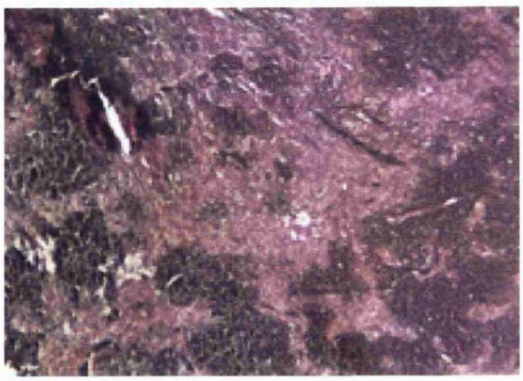
Figure 9. Genotypes and ages at death of mice that developed tumours.
U2 stands for deletion of DMR1, M or P stands for a maternal or paternal deletion of DMR1.p53wt are mice that are wild type for the p53 gene, p53het are mice that were heterozygous for the p53 gene and p53null are p53 knockout mice. Spinal stands for a tumour of the spinal column, vaginal represents a tumour of the vagina, unknown is for an unknown cause for the mouse to be unwell, subcut. represents a subcutaneous tumour of the skin, facial represents a tumour visible on the face and lymph represents a tumour found in a lymph node.

When a selection of the spontaneous tumours were sent for pathological analysis the following results were given (Figure 10):

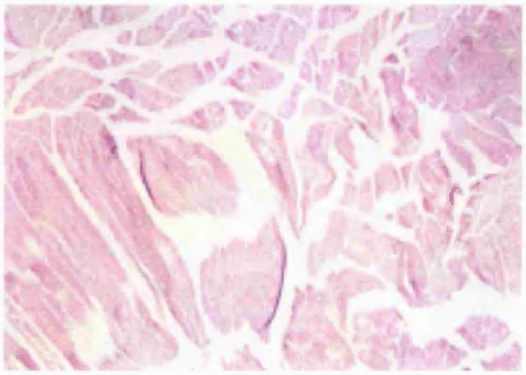
The subcutaneous tumour (2) was diagnosed as a mammary adenocarcinoma and the lymph tumour (9) was diagnosed as a malignant lymphoma, lymph tumour (7) was diagnosed as consisting of giant cells and was probably malignant.



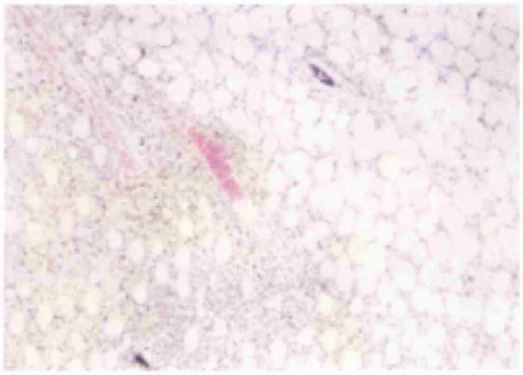
Subcutaneous tumour (2)



Lymph tumour (9)



Facial tumour (8)



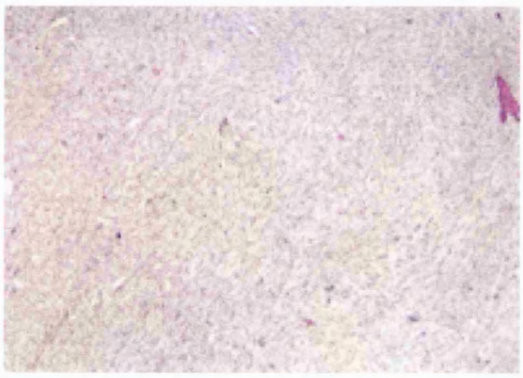
Lymph tumour (7)

Figure 10. Sections of tumours that arose spontaneously.
The sections are of H+E stained sections of spontaneous tumours at x20 magnification. The numbers in brackets were the numbers assigned to the tumours for identification purposes. (see Table 2 for details)

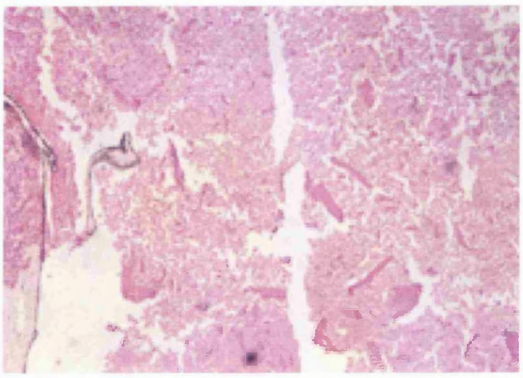
3.3.4 End of experiment analysis.

At the end of the experiment there were some mice from the sample group remaining, which had not lost any weight, did not develop spontaneous tumours and were not culled for blood and organ analysis. These mice were then culled and dissected to determine if there were any further abnormalities that could be found which were not detected previously. Paternal deletion of ΔU2 essentially has no effect and phenotypically is the same as wild type, so six of the above mice (3 wild type, 3 paternally deleted) are phenotypically the same. The one maternal deletion is associated with the *p53* wild type, with an unknown cause of weight loss and may or may not be the reason for the decrease in body mass. All of the abnormalities were found in mesodermal tissue (apart from the liver tumour which is endodermal), which corresponds with the biallelic expression of *Igf*2 in the mesoderm seen by Constancia (Constancia M. 2002). P53 het mice usually do not develop tumours until they are around 9 months old, however these mice showed no obvious abnormalities even at 20 months, more than twice the expected age. Also the mice, which were *p53*wt, showed mostly enlarged organs rather than tumour formation.

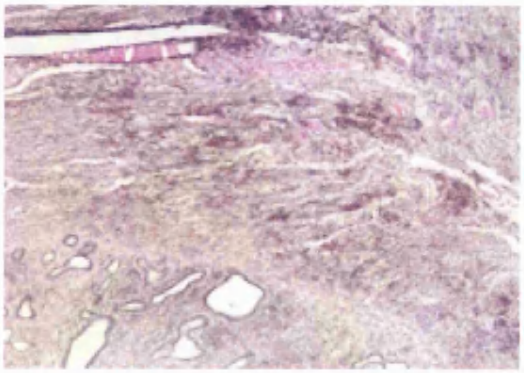
The diagnosis given when the tumours were analysed by a pathologist were as follows: Seminal vesicles (11) were described as having some areas consisting of spindle cell sarcoma, composed of fibroblastic cells with a possible diagnosis being a malignant fibrous histiocytoma. Ovarian tumour (12) was described as having two possible lesions, a large ovarian follicular cyst and a histicytic sarcoma. Ovarian tumour (13) was diagnosed as a hemangioma with thrombus formation. Facial tumour (11) was diagnosed as a hyperkeratinized papilloma.



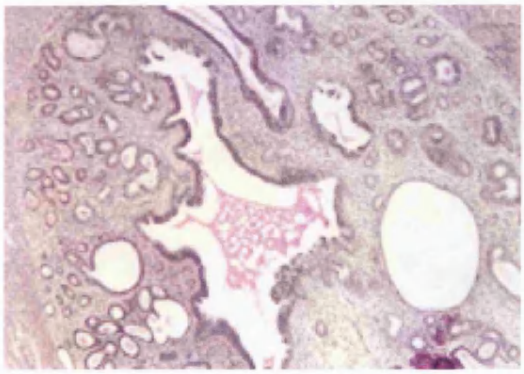
Seminal vesicles (11)



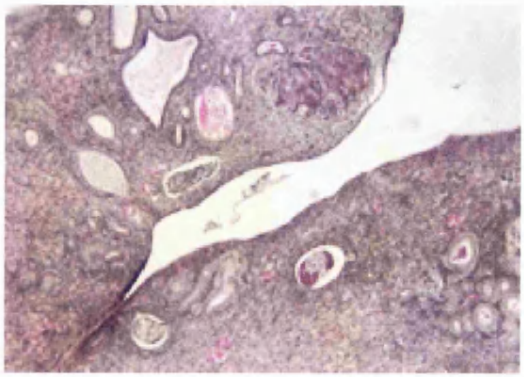
Seminal vesicles (11)



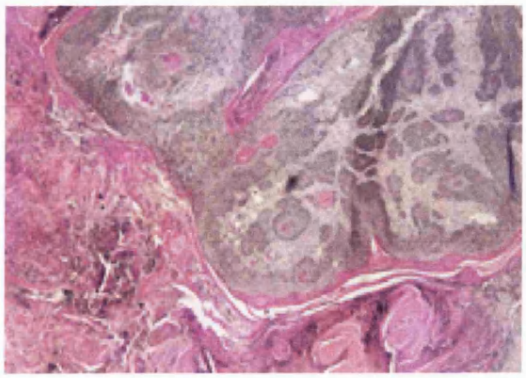
Ovarian tumour (12)



Ovarian tumour (13)



Facial tumour (11)



Uterine tumour (16)

Figure 11. Sections of tumours founding mice at the end of the experiment.
The sections are of H+E stained slides at X20 magnification. The numbers in brackets represent the numbers assigned to the tumours for identification purposes. (see Table 2 for details)

3.3.5 Genotypes of tumours.

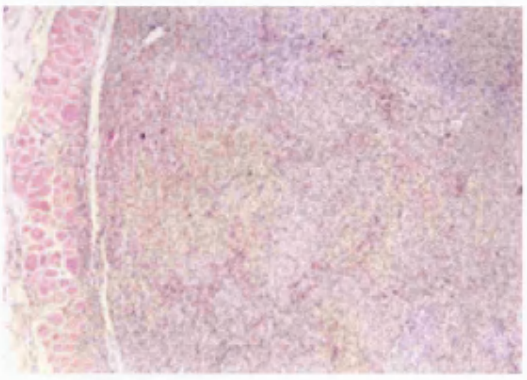
When the mice were dissected a selection of tumours were genotyped for *p53* status. (Table 2.). Ovarian tumour (12) and uterine tumour (16) both appear to have gained an allele, or rather, there has been a duplication event resulting in a potential UPD for *p53*. Ovarian tumour (13) has lost an allele in its development and it is notable that all of these changes in genotyping status occurred in the female reproductive system. Some of the tumours were genotyped for *p53* independently of their host mouse by Tracey Crew and it was found as shown above that the tumour appears to have lost an allele of *p53*. This is not unusual in tumour development and fits with Knudson's and Strongs two hit model of carcinogensis. This states that both alleles of a gene must be lost in order for cancer to develop. A mouse that is heterozygous for *p53* already has a predisposition to tumour formation and only requires one more mutation to develop cancer. Also this mouse developed cancer relatively early at 6 months which again fits with this model as mice that had two intact copies of the *p53* gene would take longer to develop the double allele loss required for tumour formation and so it would have occurred later in development.

Organ	**Genotype of mouse**	**P53 status of tumour**
End of experiment tumours		
Seminal vesicle tumour (11)	ΔU2hetM/p53het (+/-)	P53het (+/-)
Seminal vesicle tumour (10)	ΔU2hetM/p53wt (+/+)	P53wt (+/+)
Ovarian tumour (12)	ΔU2hetM/p53het (+/-)	P53wt (+/+)
Ovarian tumour (13)	ΔU2hetM/p53wt (+/+)	P53het (+/-)
Liver tumour (14)	ΔU2hetM/p53het (+/-)	P53het (+/-)
Uterine tumour (16)	ΔU2hetM/p53het (+/-)	P53wt (+/+)
Spontaneous tumours		
Subcutaneous tumour (2)	ΔU2hetP/p53het (+/-)	P53het (+/-)
Spinal tumour (4)	ΔU2hetP/p53het (+/-)	P53het (+/-)
Facial tumour (7)	ΔU2hetM/p53het (+/-)	P53het (+/-)
Lymph tumour (9)	ΔU2hetM/p53het (+/-)	P53het (+/-)
Spinal tumour (3)	ΔU2hetM/p53het	Not done
Facial tumour (8)	ΔU2hetM/p53het	Not done
Weight loss tumours		
Thymus tumour (1)	ΔU2hetP/p53het (+/-)	P53 null (-/-)
Thymus tumour (5)	ΔU2hetP/p53null	Not done
Thymus tumour (6)	ΔU2hetP/p53null	Not done
Thymus tumour (15)	ΔU2wt/p53null	Not done
Enlarged spleen (19)	ΔU2wt/p53null	Not done

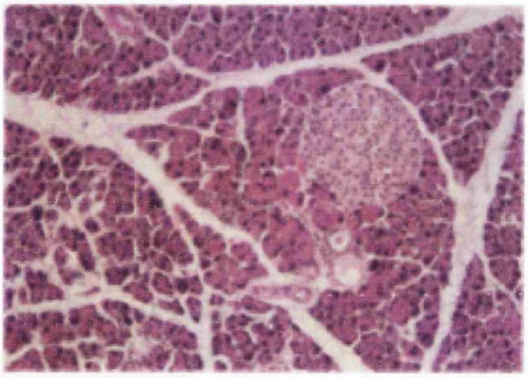
Table 2. Genotypes of mice and of tumours.
ΔU2het refers to the DMR1 status of the mice with either a maternal (M) or paternal (P) deletion of DMR1. The p53 status of the mice and tumours is represented with het short for heterozygous, wt for wild type and null for full knock out.

3.3.6 Mice culled for analysis

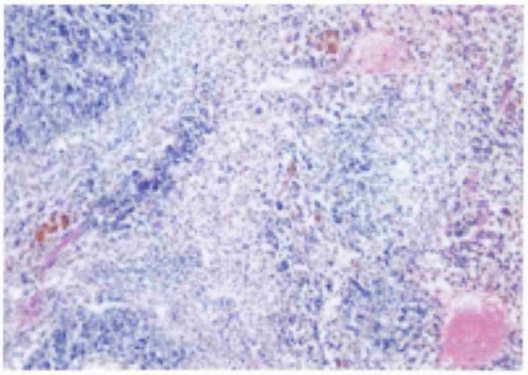
There were 4 genotypes in this group of culled mice. They were:
ΔU2 hetF/P53null, ΔU2 hetF/P53het, ΔU2WT/P53null and ΔU2WT/P53. All were culled between 9 and 10 months of age. These mice were culled in order to weigh their organs and take blood samples to measure glucose levels. A tumour which was found when the mice were dissected was sent for pathological analysis (Figure 12) and was diagnosed as a mastocytoma.



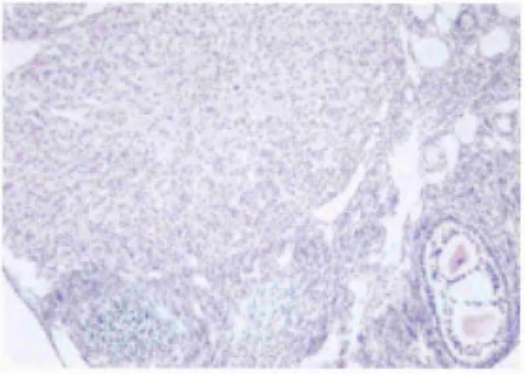
Skin tumour (20)



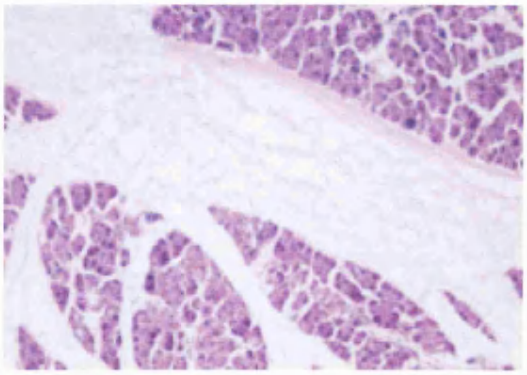
Pancreas (2)



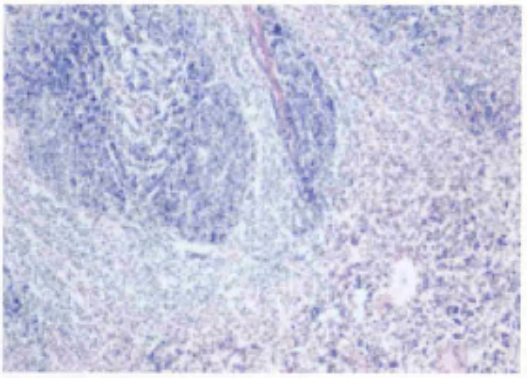
Spleen (3)



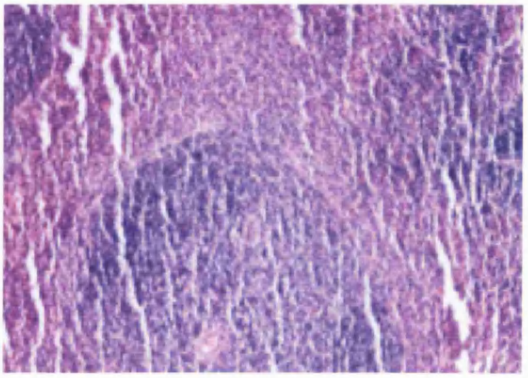
Fat (5)



Pancreas (4)



Spleen (1)



Spleen (10)

Figure 12. Sections of organs from mice culled at 9-10 months for organ weight analysis.
The sections were stained with H+E and shown here at X20 magnification. The numbers in brackets represent numbers assigned to the tumour or organs for identification purposes (see Table 2 for details)

3.4 Whole body weights and organ weights

In section 1.3 mice were culled for organ and blood analysis at 9-10 months. A. Ward also culled mice at 4 weeks and 8-12 weeks of age. Selections of organs were weighed from both of these groups of mice (divided into male and female) and blood samples were sent for glucose analysis from the mice culled at 9-10 months.

3.4.1, 4 week and 8-12 week data

Whole body weights were measured in both female and male mice at 4 weeks and at 8-12 weeks (Figure 13 and 14). Stastical analysis showed no significant difference between ΔU2hetM/p53wt and ΔU2hetP/p53wt (p= 0.352) so they were added together on the female graph. The numbers in the other categories were too low for stastical analysis.

Figure 13.

Figure 13. Female whole body weights at 4 weeks and 8-12 weeks of age.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene is represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 14.

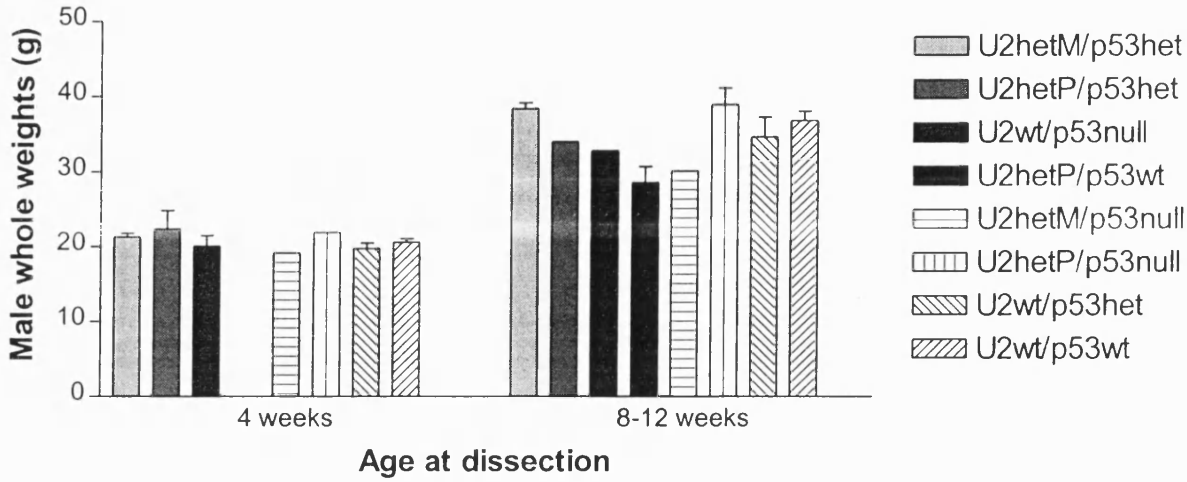


Figure 14. Male whole body weights at 4 weeks and 8-12 weeks of age.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Female and male heart weights are shown in Figure 15 and Figure 16. At 4 weeks the genotype with the heaviest hearts in the females were theΔU2wt/p53wt mice and with the lightest hearts were the ΔU2hetP/p53null mice. At 8-12 weeks there was no real difference in the heaviest heart weights between the ΔU2hetP/p53wt and the ΔU2wt/p53wt groups. The lightest hearts were in the ΔU2wt/p53null group but only just. In the male group at 4 weeks the heaviest hearts were from the ΔU2hetP/p53null and the lightest hearts were from the ΔU2hetM/p53wt group. At 8-12 weeks the heaviest hearts were in the ΔU2hetP/p53null group and the lightest hearts were in the ΔU2hetP/p53wt group.

Female and male liver weights are shown in Figure 17 and 18 respectively. At the 4-week stage, the female genotype with the heaviest livers were the ΔU2wt/p53hets and with the lightest livers were the ΔU2hetM/p53null mice. At the 8-12 week stage this changed to the ΔU2wt/p53wt mice having the heaviest livers and the ΔU2wt/p53het mice having had the lightest livers. In the male group at 4 weeks the heaviest livers were found

in the ΔU2hetP/p53null mice and the lightest in the ΔU2hetM/p53null mice. At the 8-12 week stage this was maintained.

Female and male kidney weights are shown in Figure 19 and 20 respectively. At 4 weeks in the female group the heaviest kidneys were found in the ΔU2wt/p53wt mice and the lightest in the ΔU2hetM/p53null mice. At 8-12 weeks the heaviest kidneys were in the ΔU2hetP/p53wt mice and the lightest in the ΔU2wt/p53het mice. In the male group the heaviest kidneys were in the ΔU2hetM/p53het mice and the lightest in the ΔU2hetP/p53null mice. At the 8-12 week stage both the ΔU2hetP/p53het and ΔU2hetP/p53null mice had the heaviest kidneys with the ΔU2hetM/p53null mice having the lightest kidneys.

Figure 15.

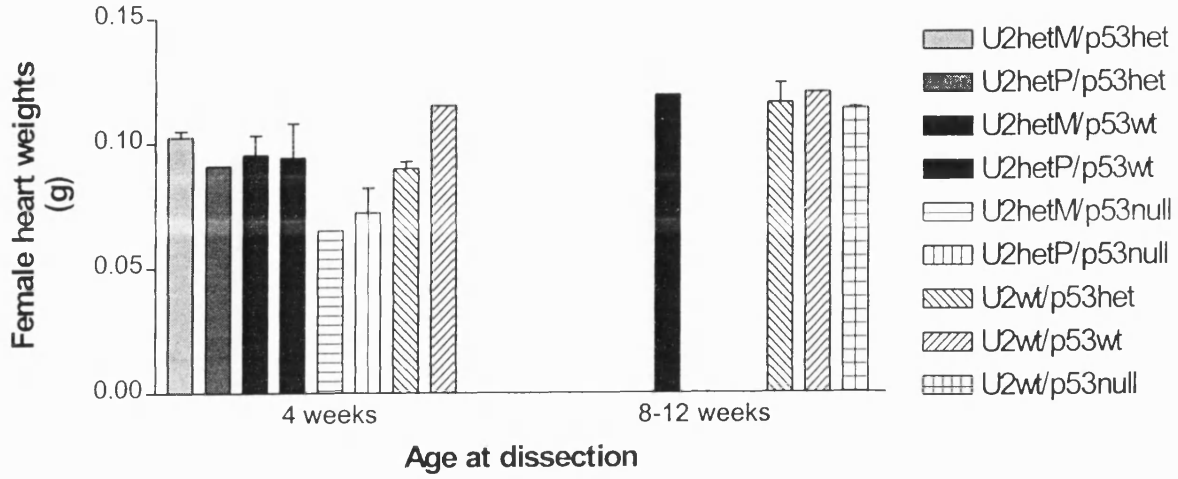


Figure 15. Female heart weights at 4 weeks and 8-12 weeks of age.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 16.

Figure 16. Male heart weights at 4 weeks and 8-12 weeks of age.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 17.

Figure 17. Female liver weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 18.

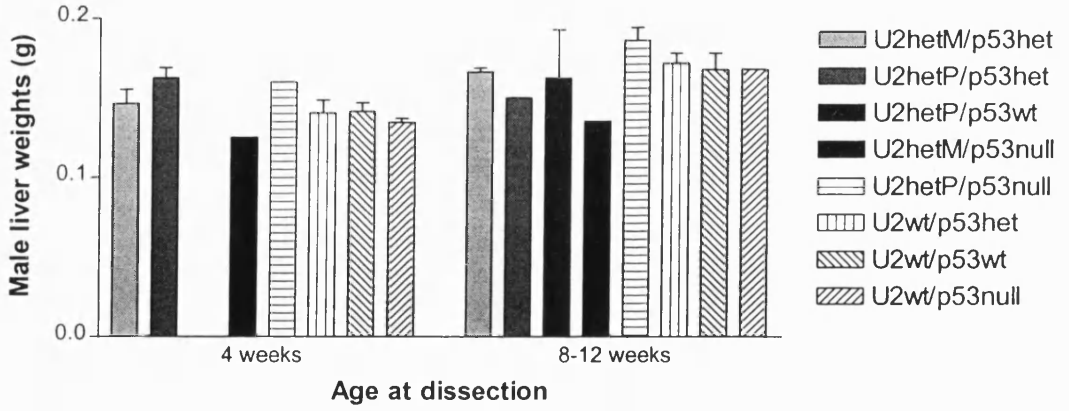


Figure 18. Male liver weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 19.

Figure 19. Female kidney weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 20.

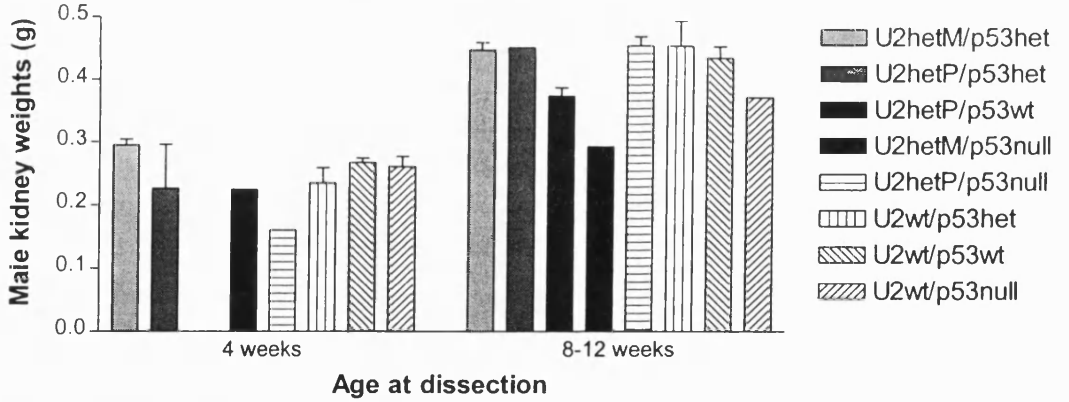


Figure 20. Male kidney weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Female uterus weights are shown in Figure 21. At 4 weeks, the heaviest uterus was in the ΔU2hetM/p53wt genotype and the lightest uterus was in the ΔU2hetM/p53null genotype. At 8-12 weeks this changed to the heaviest uterus being in the ΔU2hetP/p53wt group and the lightest uterus in the ΔU2wt/p53null group of mice.

Male testes weights are shown in Figure 22. At the 4-week stage, the heaviest testes were found in the ΔU2hetM/p53null mice and the lightest testes were found in the ΔU2hetP/p53null mice. At 8-12 weeks the heaviest testes were found in the ΔU2wt/p53null mice and the lightest testes in the ΔU2hetM/p53null mice.

Female and male thymus weights are shown in Figure 23 and Figure 24 respectively. In the female mice dissected at 4 weeks the heaviest thymus weight were observed in the ΔU2hetM/p53null mice and the lightest thymus weights were observed in the ΔU2wt/p53wt mice. At 8-12 weeks the heaviest thymus weight observed were from the ΔU2wt/p53null mice and the lightest thymus weights were from the ΔU2wt/p53het mice. In the male mice, the heaviest thymus weights observed at 4 weeks was in the ΔU2hetP/p53null mice and the lightest thymus weight was observed in the ΔU2hetM/p53null mice. At 8-12 weeks, the heaviest thymus weight observed remained in the ΔU2hetP/p53null mice and the lightest thymus weight observed was in the ΔU2hetP/p53wt mice.

Figure 21.

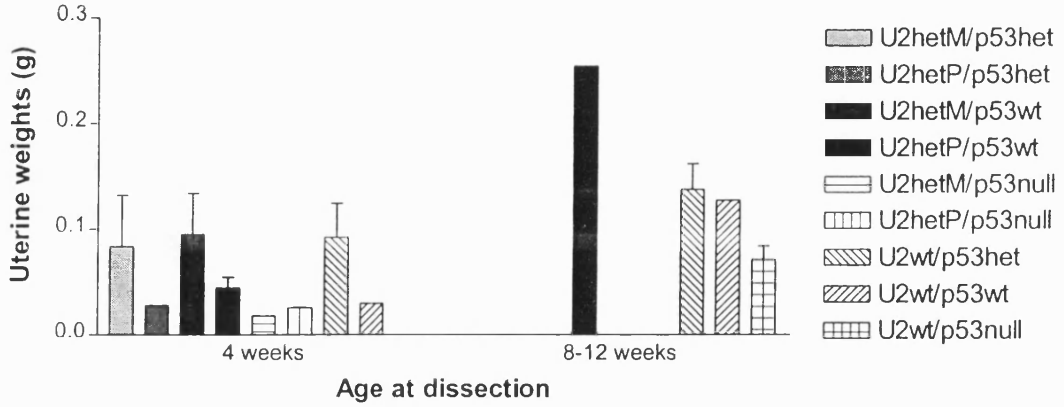


Figure 21. Female uterine weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 22.

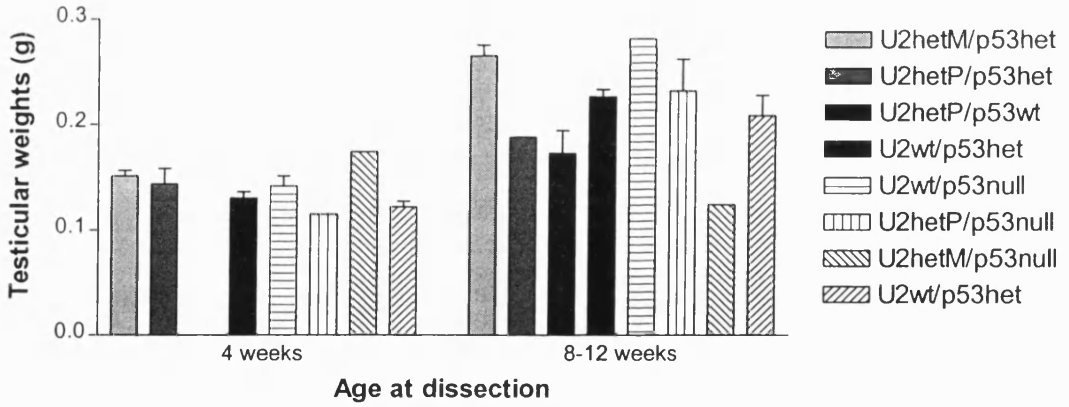


Figure 22. Male testicular weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 23.

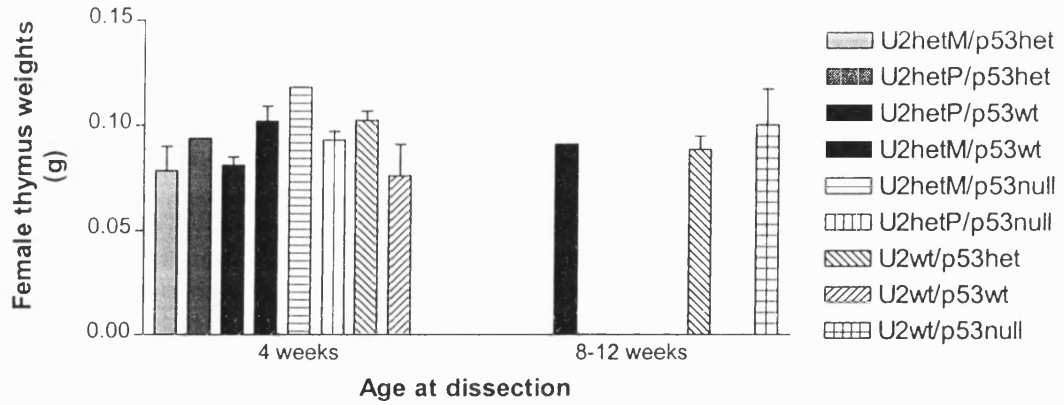


Figure 23. Female thymus weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

Figure 24.

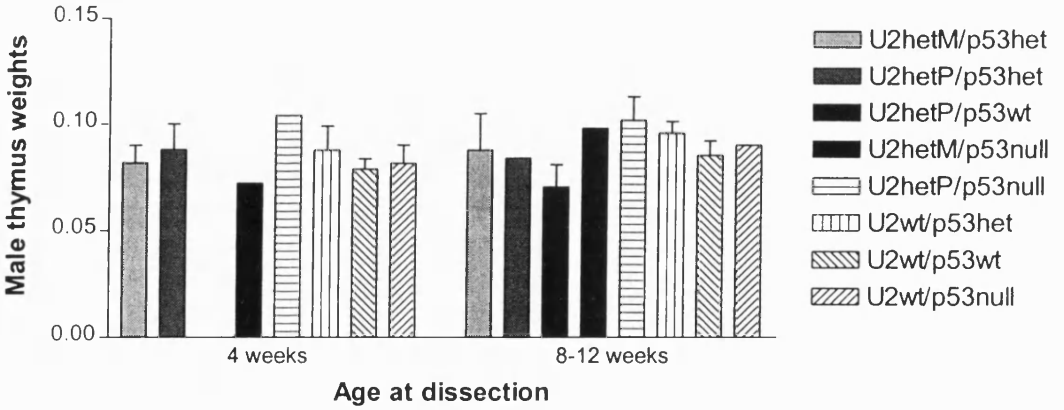


Figure 24. Male thymus weights at 4 weeks and 8-12 weeks.
A maternal deletion of DMR1 was represented by U2hetM, a paternal deletion of DMR1 by U2hetP and wild type DMR1 expression by U2wt. The status of the p53 gene was represented by heterozygous expression as p53het, wild type expression as p53wt and a full knock out by p53null.

3.4.2: 10 months organ weights and blood analysis.

At 9-10 months of age, whole body and organ weights were taken from 44 mice in the experimental group. Four genotypes were represented in this group, ΔU2hetM/p53null, ΔU2hetM/p53het, ΔU2wt/p53null and ΔU2wt/p53het. Female and male whole body weights, and female and male liver weights are shown in Figure 26. In the female whole body weights graph the heaviest genotype was the ΔU2wt/p53null group and the lightest was the ΔU2hetM/p53het group. This was also true for the female liver weights. The ΔU2wt/p53het group. Again this was maintained in the male livers.

In Figure 27 female and male spleen and brain weights are shown. The heaviest female spleen was observed in the ΔU2wt/p53null genotype and the lightest spleen was observed in the ΔU2hetM/p53het genotype. This pattern was repeated in the female brains. The heaviest male spleen was observed in the ΔU2wt/p53null genotype and the lightest male spleen was observed in the ΔU2wt/p53het genotype, which was again seen in the male brains.

Figure 28 shows female ovarian fat pad weights with the ΔU2wt/p53null mice having the heaviest fat pads and the ΔU2wt/p53het mice having the lightest fat pads. In Figure 29 which shows the male testicular fat pad weights the heaviest observed were in the ΔU2wt/p53null mice and the lightest were in the ΔU2hetM/p53null mice.

Figures 30 and 31 show female and male renal fat pad weights. In the females the heaviest renal fat pads were observed in the ΔU2wt/p53null mice and the lightest were in the ΔU2hetM/p53het mice. In the males, the heaviest renal fat pads were observed in the ΔU2wt/p53null mice and the lightest were in the ΔU2hetM/p53null mice.

Figure 26.

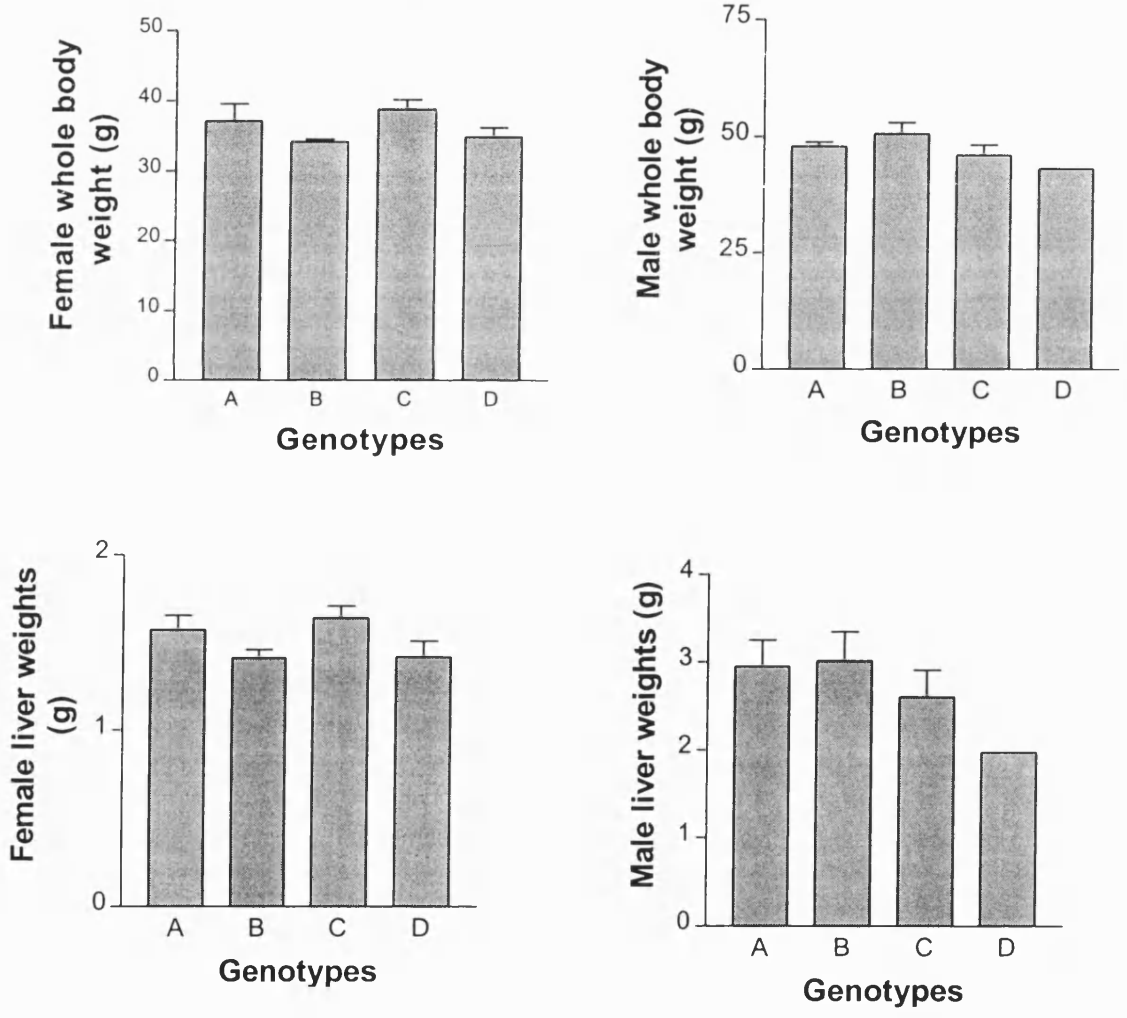


Figure 26. Female and male whole body and liver weights.
Column A represents the ΔU2hetM/p53null genotype, column B represents the ΔU2hetM/p53het genotype, column C represents the ΔU2wt/p53null genotype and column D represents the ΔU2wt/p53het genotype in all four graphs. ΔU2hetM is a maternal deletion of DMR1 and ΔU2wt is wild type DMR1. A full p53 knock out is shown by p53null and a mouse heterozygous for p53 is shown by p53het.

Figure 27.

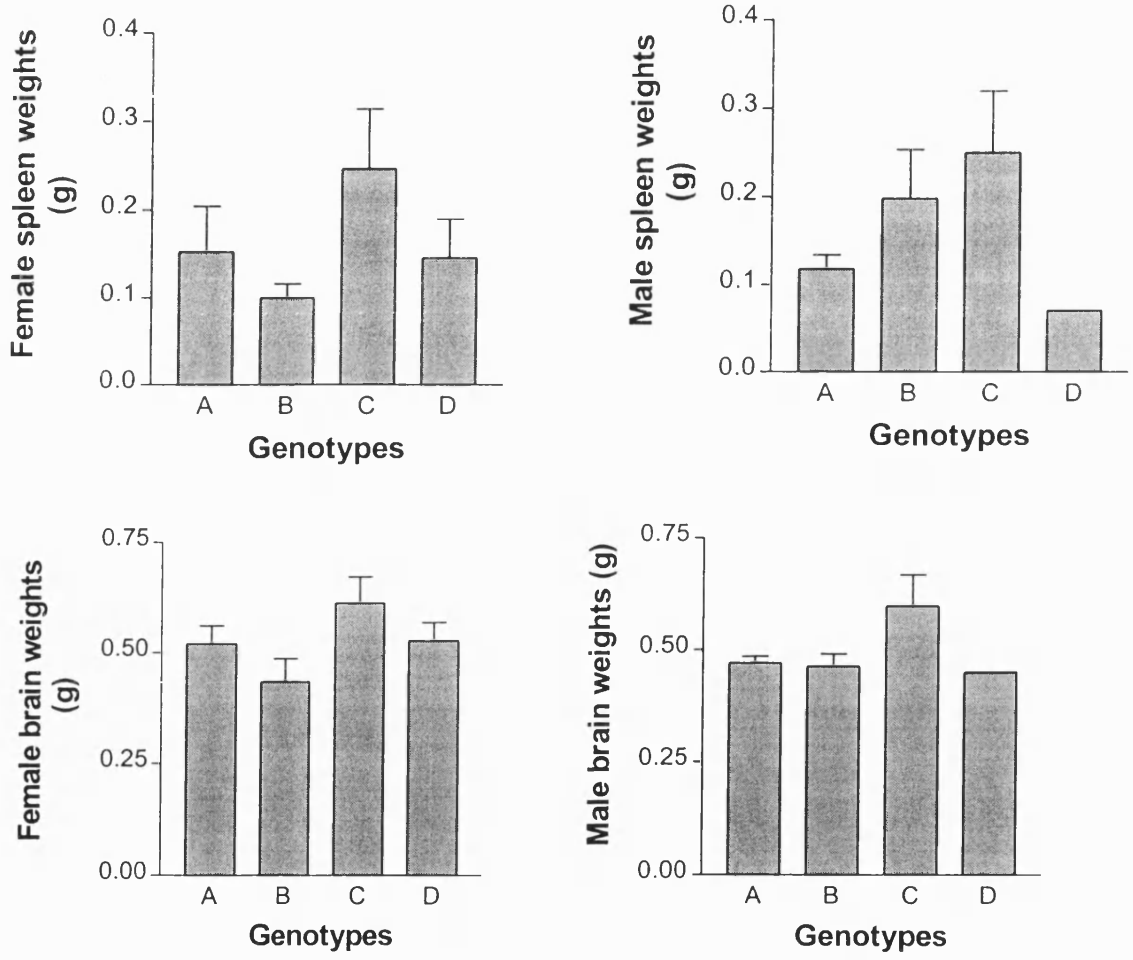


Figure 27. Female and male spleen and brain weights.
Column A represents the ΔU2hetM/p53null genotype, column B represents the ΔU2hetM/p53het genotype, column C represents the ΔU2wt/p53null genotype and column D represents the ΔU2wt/p53het genotype in all four graphs. ΔU2hetM is a maternal deletion of DMR1 and ΔU2wt is wild type DMR1. A full p53 knock out is shown by p53null and a mouse heterozygous for p53 is shown by p53het.

Figure 28.

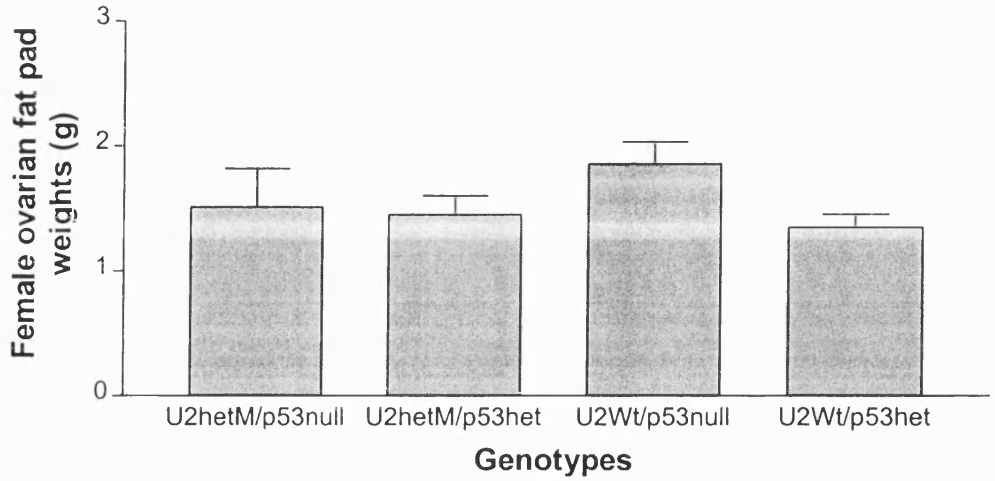


Figure 28. Female ovarian fat pad weights.
U2hetM is a maternal deletion of DMR1 and U2wt are wild type expression of DMR1. P53nulls are full knockouts of the p53 gene and p53hets are heterozygous for p53.

Figure 29.

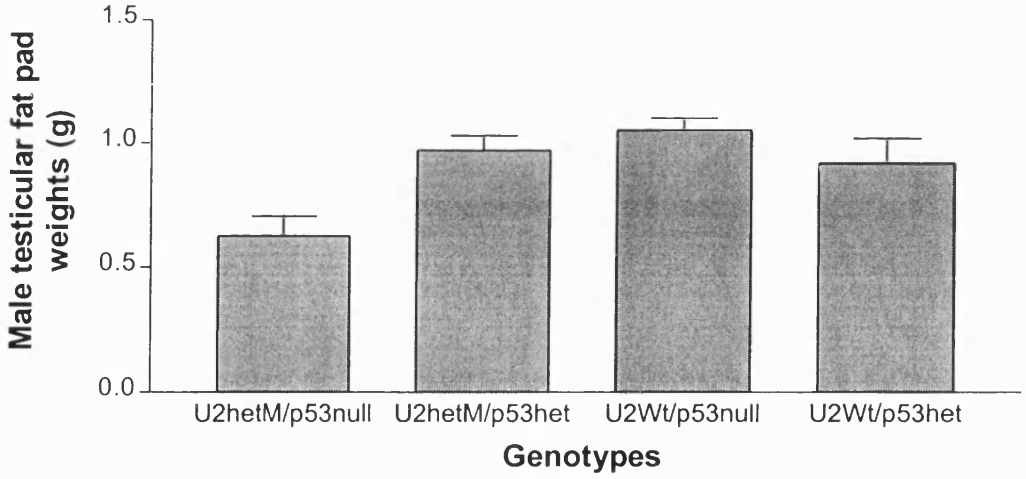


Figure 29. Male testicular fat pad weights.
U2hetM is a maternal deletion of DMR1 and U2wt are wild type expression of DMR1. P53nulls are full knockouts of the p53 gene and p53hets are heterozygous for p53.

Figure 30.

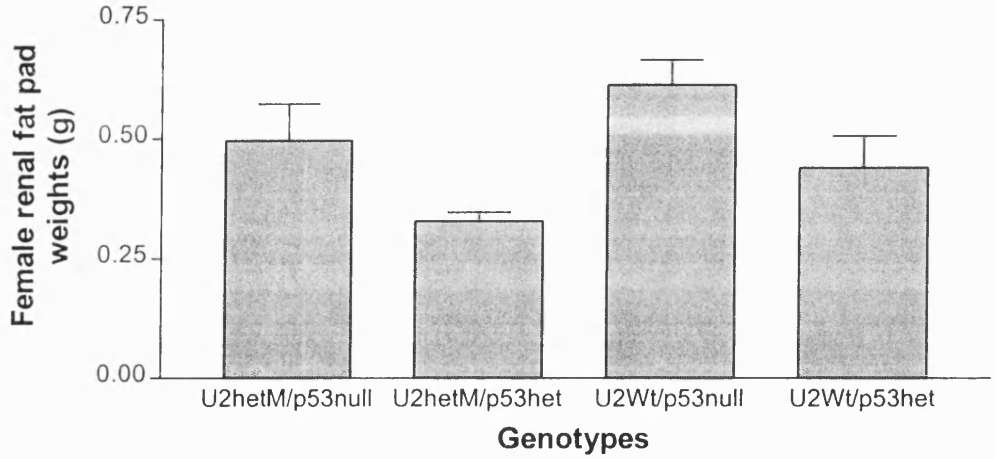


Figure 30. Female renal fat pad weights.
U2hetM is a maternal deletion of DMR1 and U2wt are wild type expression of DMR1. P53nulls are full knockouts of the p53 gene and p53hets are heterozygous for p53.

Figure 31.

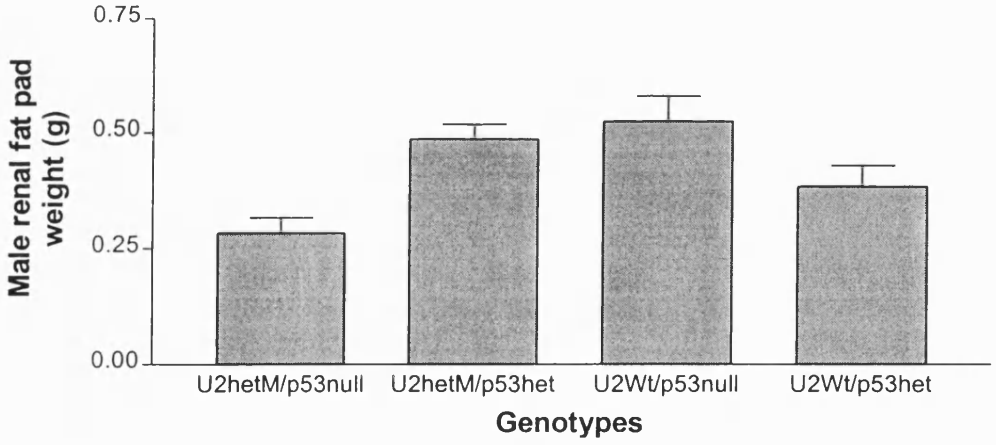


Figure 31. Male renal fat pad weights.
U2hetM is a maternal deletion of DMR1 and U2wt are wild type expression of DMR1. P53nulls are full knockouts of the p53 gene and p53hets are heterozygous for p53.

In both the female and male mice the organs were expressed as a percentage of the total body weight to detect any organs that were proportionally larger or smaller in any particular genotype (Figure 32 and Figure 33). In the female mice the uterine fat pads were the highest percentage of the total body weight whereas in the male mice the liver had the highest percentage, with the testicular fat pads coming second. No single genotype had consistently higher or lower percentages in either sex.

Figure 32.

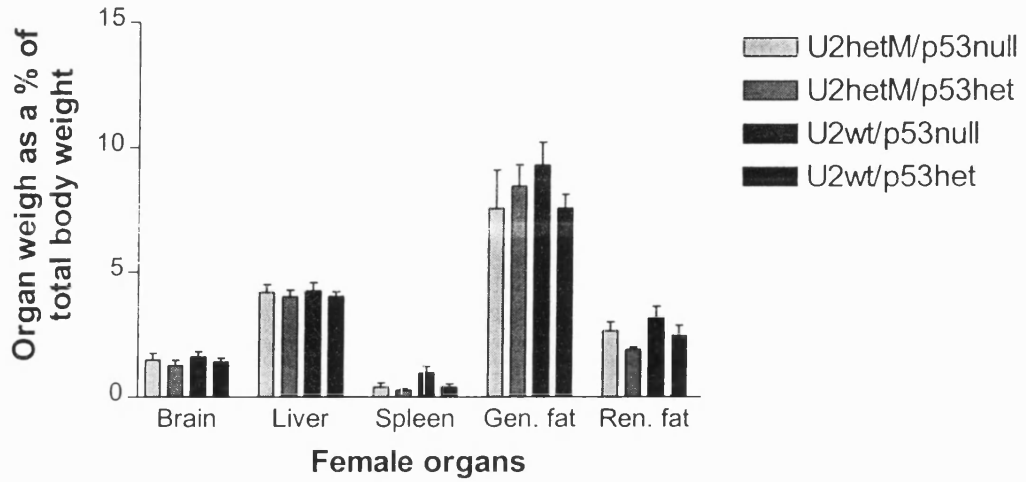


Figure 32. Female organs expressed as a percentage of the total body weight.
U2hetM is a maternal deletion of DMR1 and U2wt are wild type expression of DMR1. P53nulls are full knockouts of the p53 gene and p53hets are heterozygous for p53. The organs were the brain, liver spleen, uterine fat pads (Gen. fat) and renal fat pads (Ren. fat).

Figure 33.

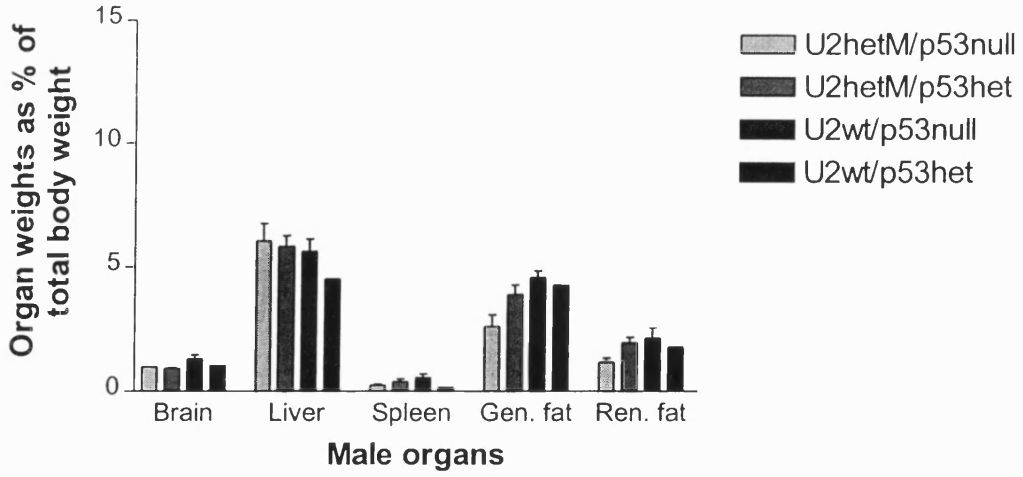


Figure 33. Male organs expressed as a percentage of the total body weight.
U2hetM is a maternal deletion of DMR1 and U2wt are wild type expression of DMR1. P53nulls are full knockouts of the p53 gene and p53hets are heterozygous for p53. The organs were the brain, liver spleen, testicular fat pads (Gen. fat) and renal fat pads (Ren. fat).

3.4.3 Growth curves.

Over a period of 12 months, all 92 mice were weighed on a weekly basis. The weights were recorded and this was also when tumours were detected and weight loss noted in any of the mice. Figure 34 shows the growth curve of some of the female mice. Not all genotypes were added to the curve as there was no significant differences in growth rate.

Figure 35 shows the rate of growth of the male mice over the same period and again not all of the genotypes were included in the graph.

Figure 34.

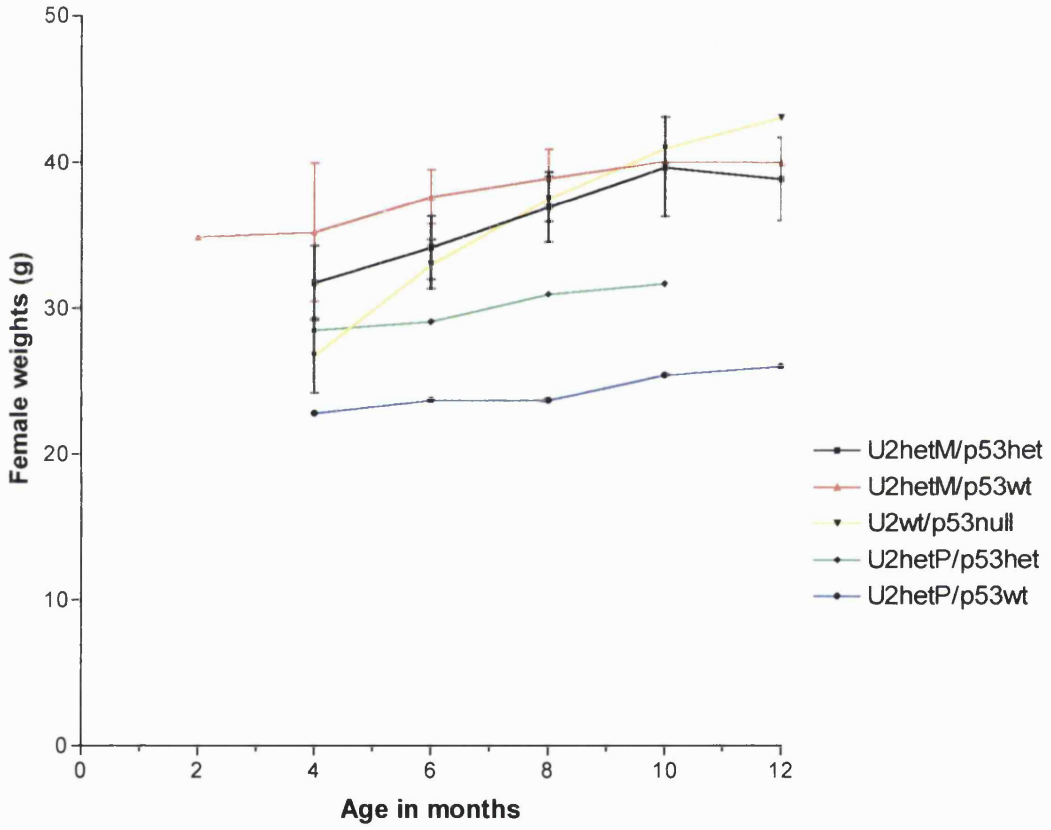


Figure 34. Effect of genotype on growth of female mice.
Maternal deletions of DMR1 are represented by U2hetM, paternal deletions by U2hetP and wild type DMR1 by U2wt. Mice heterozygous for p53 are represented by p53het, wild type p53 by p53wt and knock outs by p53null.

Figure 35.

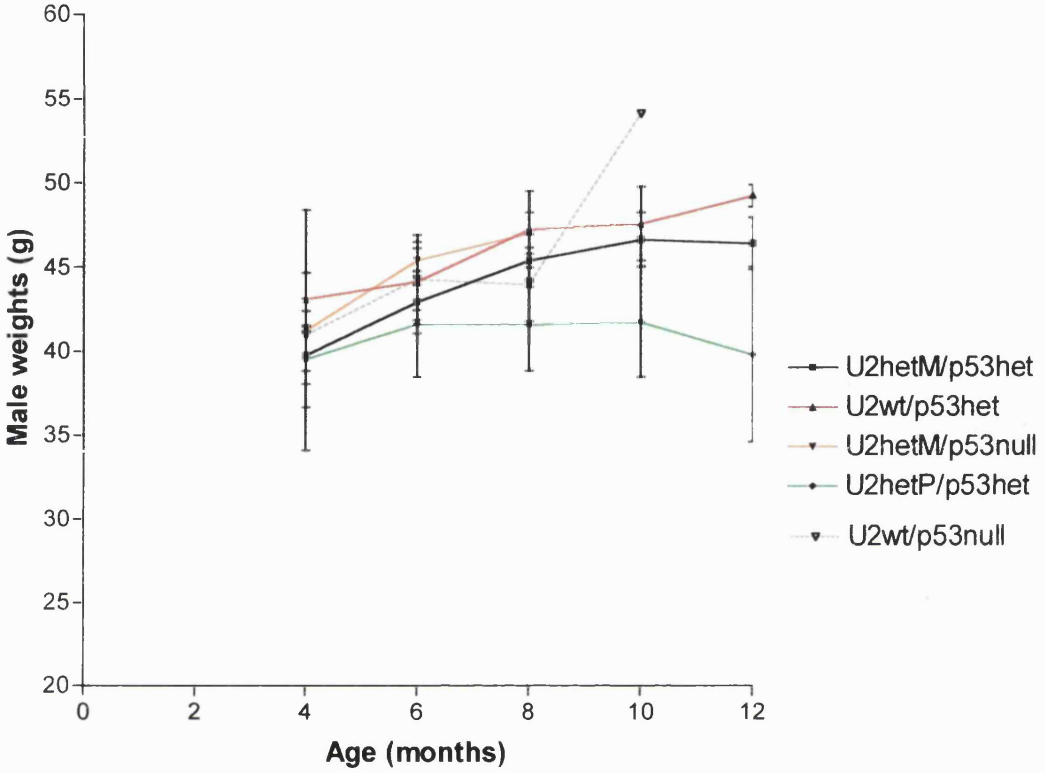


Figure 35. Effect of genotype on the growth of male mice.
Maternal deletions of DMR1 are represented by U2hetM, paternal deletions by U2hetP and wild type DMR1 by U2wt. Mice heterozygous for p53 are represented by p53het, wild type p53 by p53wt and knock outs by p53null.

3.4.4 Glucose levels analysis

The aortic blood samples taken from the mice culled for analysis were sent off and the glucose levels measured (Figure 36.). In both sexes, the genotypes with the highest circulating glucose levels were the ΔU2wt/p53null mice.

The livers from some of these mice were stained with PAS to detect gycogen in the livers (Figure 37 and Figure 38).

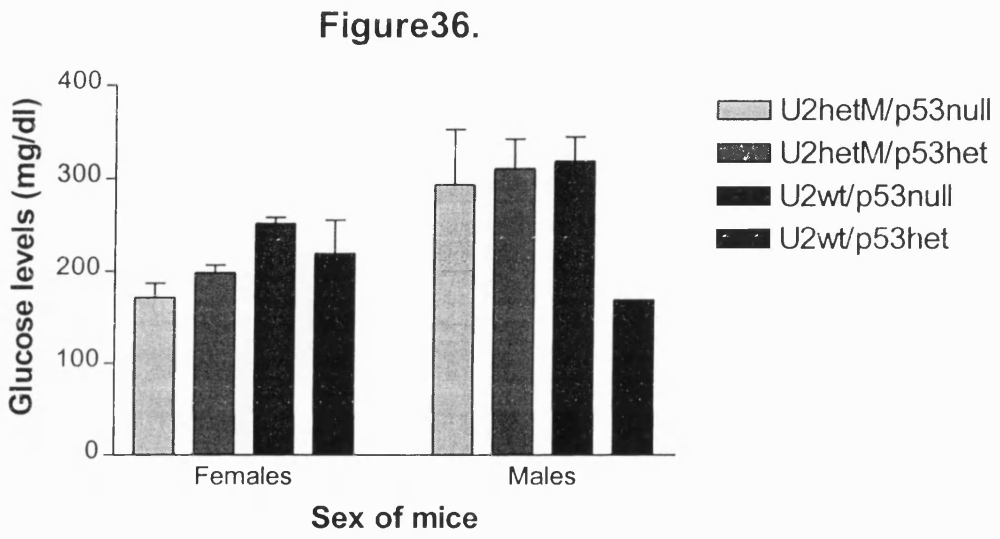
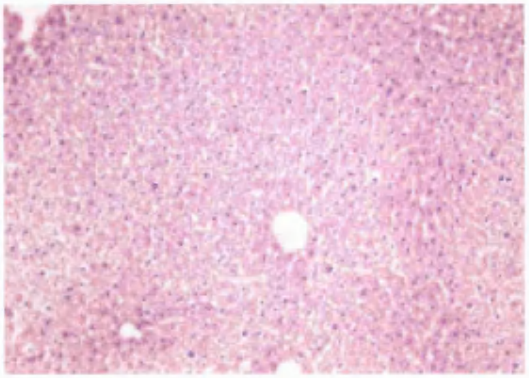
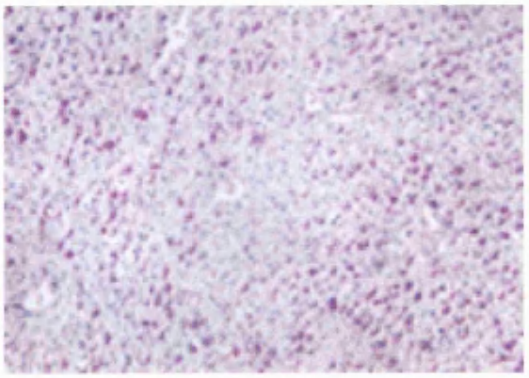


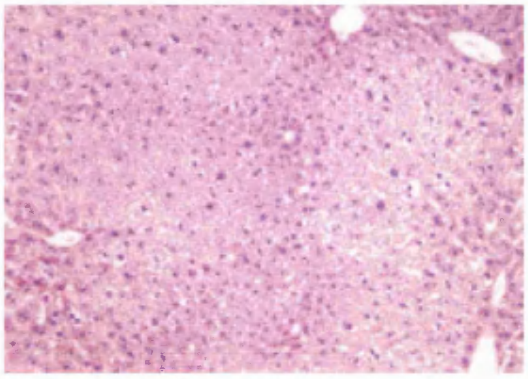
Figure 36. Average glucose levels in both sexes.
Maternal deletions of DMR1 are represented by U2hetM and wild type DMR1 by U2wt. Mice heterozygous for p53 are represented by p53het and knock outs by p53null.



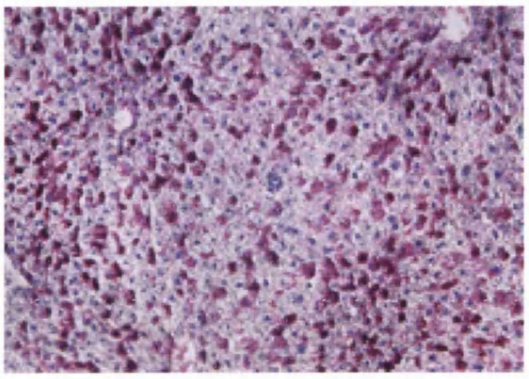
Liver (2) ΔU2het/p53het



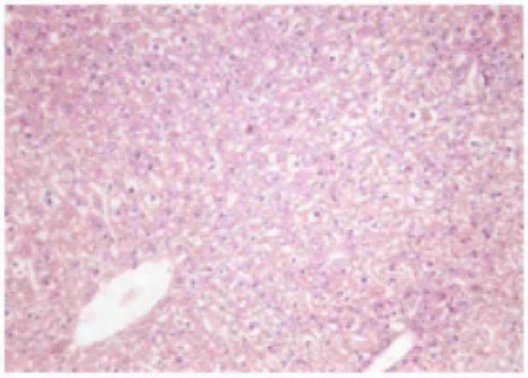
PAS stain.



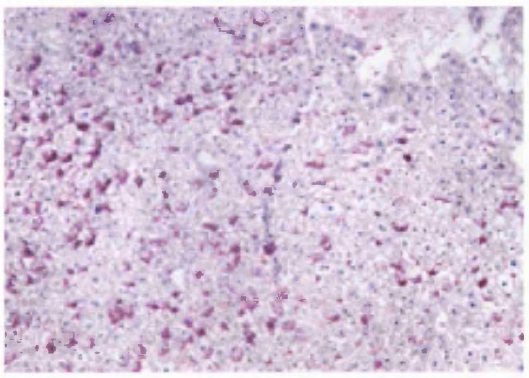
Liver (5) ΔU2wt/p53null



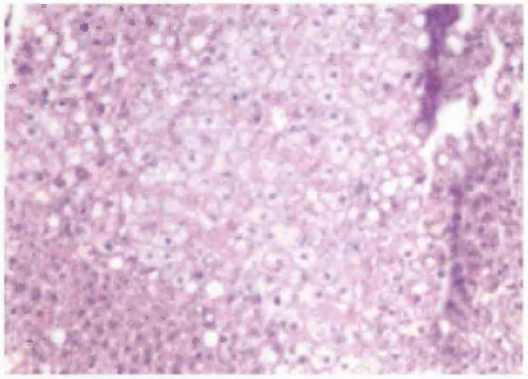
PAS stain



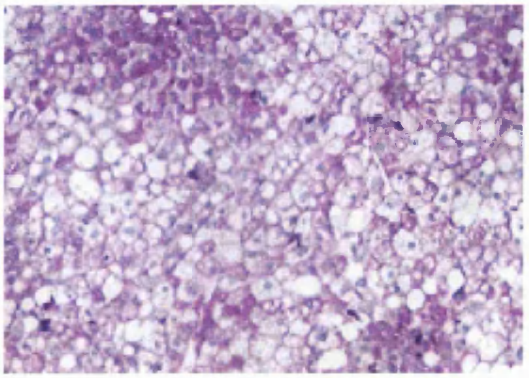
Liver (8) ΔU2hetM/p53null



PAS stain



Liver (9) ΔU2hetM/p53null



PAS stain.

Figure 37. Sections of livers taken from mice culled for analysis of organ weights.
The left panel was stained with H+E, the right panel with PAS. The numbers in brackets are numbers assigned to the organs for identification purposes.

3.3 Discussion:

The aims of this study were to investigate the combined effect of p53 tumour suppressor loss and model up-regulation of Igf2 expression knockout mice. p53 null and Igf2 ΔU2 alleles were intercrossed. From this cross 96 progeny were studied, primarily in order to correlate tumour incidence within this group.

3.3.1: mice that died of unknown causes

Of the 96 mice studied, 14 died without showing any sign of ill health. When dissected (where possible) no obvious cause of death was found and no significant weight loss had been detected prior to death. The most prevalent genotype represented in this group were ΔU2hetM/p53het mice (three of these mice died of unknown causes). In addition, one mouse of genotype ΔU2hetM/p53wt, making 4 mice in total, which had a maternal deletion of DMR1. There were 45 mice in total in this group that had a maternal deletion of DMR1, so 4 out of 45, only represents 8%, of mice dying for unknown reasons and it is unclear what the significance of maternal deletion of the normally silent DMR1.

One ΔU2hetP/p53het mouse and 3 ΔU2hetP/p53null mice died of unknown causes resulting in a total of 4 mice with a paternal deletion of DMR1 in this group. There were 16 mice in total that had a paternal deletion of DMR1 and 4 out of 16 (25%) is a large proportion of this genotype dying of unknown reasons. A paternal deletion of the normally expressed DMR1 appears to have an effect on the death rate of these mice.

Six mice in total which were wild types for DMR1 died of unknown causes. There were 25 mice in total that were wild type for DMR1 and 6 out of 25 (>33%) is a significant proportion of this genotype to die of unknown causes. Wild type DMR1 mice would not be expected to die for unknown reasons and it is possible that in this genotype the p53 status has a significant effect on the death rate in this group. However 4 of these mice are heterozygous for p53 and 2 are p53 knock outs both of which are linked with cancer development and early death in mice (Donehower 1992; Clarke 1993). Eight mice that were heterozygous for p53 out of 52 (15%) died of unknown causes, five mice that were p53 knock outs of 34 (14%) died, and only one p53wt mouse out of 10 (10%) died of unknown reasons. The p53wt mouse was as mentioned above, was ΔU2hetM, which could have been the cause of death. All of these percentages are significant numbers of a particular genotype to die for unknown reasons.

The age at death of these mice ranged from 3 to 19 months with the majority dying after 12 months of age. Loss of DMR1 does not appear to bring forward or delay that age at

death observed in mice with altered p53 in other experiments (Clarke 2003). Previous experiments showed p53 mutant mice dying at 11 months on average.

3.3.2: Mice that lost more than 10% of their total body weight in one week.

Mice that had lost greater than 10% of their total body weight in one week were culled and dissected in an attempt to explain the sudden weight loss. Ten mice in total out of the 82 remaining mice, (i.e.: exclusively those that died of unknown causes), showed this rapid weight loss. Seven of these ten mice had thymus tumours, one had an enlarged spleen and two mice had no detectable pathological change associated with the weight loss.

Pathological analysis of some of these abnormal organs showed a malignant lymphoma for thymus no.1 (ΔU2hetM/p53het) and thymus no. 5 (ΔU2hetM/p53null) and an enlarged cellular structure for spleen no. 19 (ΔU2wt/p53null).

Thymus tumours are not unusual for mice with altered p53 and an enlarged spleen can be an indication of infection rather than any genetic cause. Research carried out by Donehower (Donehower 1992) showed that the most frequently observed tumours found in p53-altered mice were malignant lymphomas usually in the thymus and other visceral organs including the spleen.

The most prevalent genotype among of mice that lost >10% of their total body weight in one week, were the ΔU2wt/p53null (71%)mice which is to be expected as p53 null mice are most likely to develop lymphomas. In the analysis carried out by Donehower 26 out of the 30 mice analysed were p53nulls found to have lymphomas.

The mouse that had an unknown reason for their weight loss was p53wt .It is possible that the weight loss was not due to tumour formation but some other etiological factor.

Most of the weight loss in these mice was detected at ages 5 to 10 months, with most of the thymus tumours detected at 5 to 6 months of age. The enlarged spleen was detected at 6 months and the unattributed weight loss occurred at 10 months. P53 null mice are prone to neoplasm development at 6 months of age (Donehower 1992), and in this study 7 out of the 10 mice with weight loss were p53null mice.

3.3.3: Tumour development.

Nine mice out of the 96 mice studied developed obvious abnormalities in the absence of a greater than 10% body weight loss. Out of the six abnormalities found (spinal, lymph, facial, subcutaneous, vaginal and leg) most were tumours that affected the lymph nodes. The range of tumours types was more extensive than in the mice with a detectable weight loss.
The genotypes of the abnormalities were mostly ΔU2hetM/p53het. The subcutaneous tumour was also a p53het but with a paternal deletion of DMR1. Only one of the lymph node tumours was ΔU2hetP/p53null. Lymph node tumours have been detected in previous studies (Donehower 1992),(Clarke 2003) and are not unusual in mice with altered p53 expression. Pathology reports on lymph node tumour no.7 and no.9 showed probable malignancy, with multinuclear giant cells.

The facial tumour was unusual with no previous facial tumour mentioned in other similar studies. However, it is likely that this was a sarcoma, and sarcomas are common in p53 mutant mice.

Subcutaneous tumour no.2 was diagnosed as a mammary adenocarcinoma. The Donehower 1992 study included one mouse with a mammary adenocarcinoma in the p53 null genotype, and in this study the same tumour occurred in the ΔU2hetP/p53het group. With only one mouse developing a tumour of this type it is not possible to ascribe any significance in the different genotype of this mouse and that of the Donehower (1992) study.

Genital tumours have been detected in previous studies (ovarian sarcomas, ovarian choriocarcinmoa, Leydig-cell tumours of the testis and embryonal carcinoma of the testis). However tumours of the vagina or labia are not mentioned in these studies so this tumour could possibly be outside the typical tumour spectrum. However it did not result in dissection at an unusual age and it was in a tumour-associated genotype (ΔU2hetM/p53het).

The leg abnormality found in this group did not reveal a tumour. The mouse had difficulty walking but when analysed no source was found. This could have been to a physical injury rather than a physiological or genetic abnormality.

Within this group of mice that developed spontaneous abnormalities there was no statistical difference between the genotypes regarding the ages at which they were culled. The mice were culled between the ages of 12 to 18 months. This was older than the mice that showed a detectable weight loss. When compared statistically there was a significant difference ($p = 0.0039$)(Mann-Whitney analysis). These mice mostly developed lymphomas whereas the weight loss group developed thymus tumours. It seems that weight loss can be associated with thymus tumour development and can lead to earlier detection of this tumour

type. Lymphomas do not seem to induce significant weight loss and so the mice are older by about six months when a tumour finally becomes obvious.

3.3.4: End of experiment analysis

When the mice that had lost >10% of their body weight, the mice that had developed spontaneous tumours and the mice culled for analysis of organ weights and blood glucose levels had been removed there were 19 mice left that were culled and dissected at the end of the experiment. Upon dissection, some tumours were found (Figure 11.).

Enlarged seminal vesicles were found in one mouse (seminal vesicles no. 11) which were diagnosed as a spindle cell sarcoma composed of fibroblastic cells. Again, these were seen in the Donehower (1992) study and are therefore not unusual for p53-altered mice. The same mouse also had a facial tumour (no. 11), which was described as a hyperkeratinized squamous cell papilloma. This was the only mouse in which two obvious and separate tumours were detected.

One ovarian tumour (no. 12) was diagnosed as two different lesions, an endometrial stromal sarcoma and a hysticytic sarcoma. The mouse was 20 months old when culled. Ovarian tumour (no. 13) was diagnosed as a hemangioma with thrombus formation and the mouse was 20 months old when culled. A uterine anomaly (no. 16) was thought likely to be a uterine glandular polyp. In the Donehower (1992), study a chimeric mouse at 14 months developed osteogenic sarcoma and a choriocarcinoma. This suggests that tumours of the female reproductive organs are complex and usually develop much later than the previously discussed thymus and lymph node tumours.

3.3.5 Genotypes of tumours

Some of the tumours were genotyped independently of the mice they were removed from and the results were given in Table 2. Two tumours appeared to lose a p53 allele, an ovarian tumour (no.13), which went from ΔU2hetM/p53wt to p53het (allele loss), and a thymus tumour (no.1), which went from ΔU2hetP/p53het to p53null. The ovarian tumour was detected at 20 months which is late in development, but the mouse was wild type and it may take longer for mutations to occur which change p53wt to p53het than to change p53het to p53null.

The thymus tumour (no.1) was detected earlier than 20 months. This fits with Knudson's and Strongs two hit model of carcinogenesis. This states that both alleles of a gene must be lost in

order for cancer to develop. A mouse that is heterozygous for p53 already has a predisposition to tumour formation and only requires one more mutation to develop cancer. Also this mouse developed cancer early at six months, which again fits with this model, as mice that had two intact copies of the p53 gene would take longer to develop the double allele loss required for tumour formation and so, it would occur later in development.

Two of the tumours appeared to lose the mutant p53 allele, ovarian tumour (no.12) went from ΔU2hetM/p53het to p53wt and uterine tumour (no.16) went from ΔU2hetM/p53het to p53wt.

3.3.6: Mice culled for analysis

In total 44 mice were culled for organ weight and blood glucose analysis. The ages at death were between 9 and 10 months of age. Sections were taken of the organs that were weighed and some of them were stained with H+E (see Figure 12). No abnormalities were found in these organs.

3.4.1: 4 week and 8-12 week data

Cohorts of mice were culled at 4 weeks and 8-12 weeks of age and their organs were weighed. Mice were also weighed at 9-10 months of age, their dissected organs weighed, and their blood analysed for glucose levels.

At four weeks of age in the female mice (Table 1) the genotype with the heaviest organ the majority of the time was the ΔU2wt mice, whether they were p53het or p53wt. This result agrees with the expected answer as maternal deletion of DMR1 had no effect on organ weight and paternal deletion of DMR1 led to smaller pups at birth that caught up with their wild type littermates postnatally (Constancia 2000). It appears that at 4 weeks ΔU2hetP mice had not quite caught up with ΔU2wt mice.

The genotypes associated with the lightest organs at 4 weeks were the ΔU2hetM/p53null mice. A maternal deletion of DMR1 does not increase organ weight and out of the three possible states of Igf2, maternal deletion of DMR1 could lead to lightest organ weights.

At 8-12 weeks in the female mice the heaviest organs were evenly spread between ΔU2hetP and ΔU2wt genotypes but the majority were p53wt. The accelerated growth seen in ΔU2hetP mice after birth appears to have brought these mice to the same weights as the ΔU2wt mice. The lightest organs at 8-12 weeks were associated with ΔU2wt/p53het mice. All of the lightest organs were ΔU2wt which could suggests that the ΔU2hetP mice have overtaken the ΔU2wt mice.

Organ (♀)	4 wk. heaviest	4 wk. lightest	8 wk. heaviest	8 wk. lightest
Whole body	ΔU2wt/p53het	ΔU2hetM/p53null	ΔU2het/p53wt	ΔU2wt/p53wt
Heart	ΔU2wt/p53wt	ΔU2hetP/p53null	ΔU2wt/p53wt	ΔU2wt/p53null
Liver	ΔU2wt/p53het	ΔU2hetM/p53null	ΔU2wt/p53wt	ΔU2wt/p53het
Kidney	ΔU2wt/p53wt	ΔU2hetM/p53null	ΔU2hetP/p53wt	ΔU2wt/p53het
Uterus	ΔU2hetM/p53wt	ΔU2hetM/p53null	ΔU2hetP/p53wt	ΔU2wt/p53null
Thymus	ΔU2hetM/p53null	ΔU2wt/p53wt	ΔU2wt/p53null	ΔU2wt/p53het

Table 1. Summary of heaviest and lightest organ weights at 4 weeks and 8 weeks of age in female mice. ΔU2 wt are mice with wt DMR1, ΔU2hetM have a maternal deletion of DMR1 ΔU2hetP have a paternal deletion of DMR1. p53het are mice that are heterozygous for p53, p53 null are p53 knockout mice and p53wt are mice with wild type expression of p53.

In the male mice (Table 2) at 4 weeks of age, the heaviest genotypes were the ΔU2hetP/p53null mice. The accelerated growth associated with paternal deletions of DMR1 appears to be evident here, to the extent that the ΔU2hetP mice are heavier than their ΔU2wt. The lightest genotypes were the ΔU2hetM/p53null mice. However, all of these mice were p53nulls.

At 8-12 week stage of development, the mice with the heaviest organs were the ΔU2hetP/p53null mice. Again, the catch-up effect of paternal deletion of DMR1 seems to be out performing the ΔU2wt mice. The lightest organs were evenly distributed between the ΔU2hetP/p53wt and ΔU2hetM/p53null mice.

Organ (♂)	4 wk. heaviest	4 wk. lightest	8 wk. heaviest	8 wk. lightest
Whole body	ΔU2hetP/p53het	ΔU2hetM/p53null	ΔU2hetP/p53null	ΔU2hetP/p53wt
Heart	ΔU2hetP/p53null	ΔU2hetM/p53wt	ΔU2hetP/p53null	ΔU2hetP/p53wt
Liver	ΔU2hetP/p53null	ΔU2hetM/p53null	ΔU2hetP/p53null	ΔU2hetM/p53null
Kidney	ΔU2hetM/p53null	ΔU2hetP/p53null	ΔU2wt/p53het	ΔU2hetM/p53null
Testes	ΔU2hetM/p53null	ΔU2hetP/p53null	ΔU2wt/p53null	ΔU2hetM/p53null
Thymus	ΔU2hetP/p53null	ΔU2hetM/p53null	ΔU2hetP/p53null	ΔU2hetP/p53wt

Table 2. Summary of heaviest and lightest organ weights at 4 weeks and 8 weeks of age in male mice. ΔU2 wt are mice with wt DMR1, ΔU2hetM have a maternal deletion of DMR1 ΔU2hetP have a paternal deletion of DMR1. p53het are mice that are heterozygous for p53, p53 null are p53 knockout mice and p53wt are mice with wild type expression of p53.

3.4.2: 9-10 months organ weight analysis

In the female mice at 9-10 months (Table 3) of age the heaviest genotype was the ΔU2wt/p53null mice; illustrating that wild type DMR1 mice have heavier organs than mice with altered DMR1 expression. The lightest organs were associated with ΔU2hetM/p53het mice in the majority of mice analysed. A maternal deletion of DMR1 leading to altered Igf2 expression leads to lighter organs than those observed in ΔU2wt mice.

Organ (♀)	Heaviest genotype	Lightest genotype
Whole body weight	ΔU2wt/p53null	ΔU2hetM/p53het
Liver	ΔU2wt/p53null	ΔU2hetM/p53het
Spleen	ΔU2wt/p53null	ΔU2hetM/p53het
Brain	ΔU2wt/p53null	ΔU2hetM/p53het
Ovarian fat pad	ΔU2wt/p53null	ΔU2wt/p53het
Renal fat pad	ΔU2wt/p53null	ΔU2hetM/p53het

Table 3. Summary of heaviest and lightest organ weights at 9-10 months of age in female mice. ΔU2 wt are mice with wt DMR1, ΔU2hetM have a maternal deletion of DMR1 ΔU2hetP have a paternal deletion of DMR1. p53het are mice that are heterozygous for p53, p53 null are p53 knock-out mice and p53wt are mice with wild type expression of p53.

In the male mice the heaviest organs were associated with ΔU2wt/p53null mice as was observed in female mice at the same age. Unlike what was seen in females, the lightest organs were observed in ΔU2wt/p53het mice rather than ΔU2hetM/p53het mice observed in female mice.

Organ (♂)	Heaviest genotype	Lightest genotype
Whole body weight	ΔU2hetM/p53het	ΔU2wt/p53het
Liver	ΔU2hetM/p53het	ΔU2wt/p53het
Spleen	ΔU2wt/p53null	ΔU2wt/p53het
Brain	ΔU2wt/p53null	ΔU2wt/p53het
Testicular fat pad	ΔU2wt/p53null	ΔU2hetM/p53null
Renal fat pad	ΔU2wt/p53null	ΔU2hetM/p53null

Table 4. Summary of heaviest and lightest organ weights at 9-10 months of age in male mice.
ΔU2 wt are mice with wt DMR1, ΔU2hetM have a maternal deletion of DMR1 ΔU2hetP have a paternal deletion of DMR1. p53het are mice that are heterozygous for p53, p53 null are p53 knockout mice and p53wt are mice with wild type expression of p53.

When the organ weights were expressed as a percentage of whole body weight, in the females the heaviest genotypes were the ΔU2wt/p53null mice and in the male the same was true except for the liver weights where the ΔU2hetM/p53null mice had the heaviest livers.
In the female mice, the genital fat pad weights took up the largest percentage of the total body weight, but in the males the livers took up the largest percentage followed by the genital fat pad weights.

3.4.3Growth curves

In the female mice the steepest growth curve was seen in the ΔU2wt/p53null mice, which grew bigger than the ΔU2hetM/p53wt mice in the final 2 months of this study. The smallest mice were the ΔU2hetP/p53wt mice. In the male mice the steepest growth curve was seen in the ΔU2wt/p53null mice also, which overtook the ΔU2wt/p53het mice 4 months before the end of the study. The lightest mice were the ΔU2hetP/p53het mice. Paternal deletions of DMR1 result in smaller mice and DMR1 wild type mice are the largest in this group. The standardised average expected weight for a non-transgenic mouse at 12 months is around 35g

which is slightly higher than the female weight observed in this study and slightly lower than he observed male weights discussed here.

3.4.4 Glucose analysis

The results from the glucose analysis are shown in Figure 36. In both the female and male mice, the highest glucose levels were found in the ΔU2wt/p53null mice. These mice also had the heaviest organs and the highest percentages body fat. In addition, these mice had the steepest growth curves.

These mice which have high body fat ratios and high circulating glucose levels may also have relatively low circulating levels of *Igf2*. ΔU2wt mice would be expected to have higher circulating levels of *Igf2* than ΔU2hetM mice do, as there was no deletion of DMR1 (Rogler 1994) (Ward 1994). To further analyse the effect on energy storage in these mice a selection of liver sections were stained with PAS, which stains glycogen purple. The darkest purple staining can be seen in liver (5), which was from a ΔU2wt/p53null mouse and had the most glycogen on the liver.

Chapter four: Analysis of transgenic mice and transient transfections

4.1.1 Transgenics

A transgenic organism is one derived from a cell in which the genome has been modified by the addition of exogenous DNA. In these experiments, two types of reporter gene were used in constructs that were injected into mouse embryos. The luciferase reporter gene allowed quantification of the expression of the transgene and the beta-galactosidase (*lac*Z) reporter gene allowed qualitative analysis of transgene expression.

A transgenic organism can be created in a number of ways such as nuclear injection, viral infection and transfection. Yeast and plant cells can be transfected (e.g. by electroporation) with DNA, having used enzymes to remove their outer coating. The cells (spheroplasts) are then capable of taking in the exogenous genes. Yeast, plant and animal cells can be electroporated, which makes their membranes permeable to DNA in their medium (Loadish 2000). Initial experiments were carried out on the yeast *Saccharomyces cerevisiae* using plasmids. Yeast artificial chromosomes (YAC) were developed which had a centromere; telomeres and autonomous replication sequences (ARS) added and were linearised. This allowed the YACs to be treated more like yeast chromosomes when the cells divided and were passed onto the next generation of cells (Griffiths 1999). This ability that yeast has to take up foreign DNA is useful in creating knockout yeast cells. A mutant allele of a particular gene can be added to the cells, which has been altered in such a way as to not produce a protein. Any cells with this mutant gene will not be able to produce by homologous recombination, the protein of interest and so are considered knockouts (Loadish 2000).

To create transgenic plants the vector used most often came from the bacteria *Agrobacterium tumefaciens* which caused crown gall disease in plants (plant tumours). When the transgene was spliced into this Ti plasmid (tumour inducing) and this was introduced to the plant the transgene was integrated into the plant genome (Griffiths 1999).

In animals, transgenesis has been used to examine development and disease mostly in *Drosophila*, nematode worms (*Caenorhabditis elegans*) and mice. Transgenic *Drosophila* were created using a reporter gene (Section 4.1.2) and heat shock genes incorporated into the transgene construct. The flies were allowed to reach the appropriate stage of development and then were heat shocked to activate the transgene. The flies were then assayed to examine were the gene was expressed (Griffiths 1999). An example of an aberrant phenotype created using transgenics is with the gene *Antennapedia (Antp)* which

affects leg development. When this was introduced to the head region of *Drosophila*, a leg developed at the front of the head instead of antenna (Loadish 2000).

Transgenic mice are usually created by injecting foreign DNA into either the male or female pronucleus {Gordan, 1980 #68}. The resulting mice are either transgenic (the transgene is present in every cell) or mosaic (the transgene is present in a subset of cells). The zygote can then be transferred into the uterus of a host mouse and develops as normal. Where the transgene inserts into the genome is essentially random and there are usually multiple copies present at a single integration site. Up to 30% of offspring can carry the transgene which, when genotyped and identified, can be backcrossed to create heterozygous and homozygous mice which are positive for the transgene.

Knock out mice are created in a slightly different manner. A specific deletion cannot be introduced directly into an organism's germ cells so a method of deleting DNA in cells and combining those cells with wild type blastocyst stage embryos was developed. Embryonic stem (ES) cells, derived from the inner cell mass of the blastocyst are mutated and the deletion of the gene of interest is induced. Having checked for the deletion these mutated ES cells are added to wild type blastocyst preimplantation embryo cells, which are then transferred into host mothers. The resulting offspring are called chimeras as they have tissues derived from both wild type cell and the mutant ES cells. The offspring are genotyped and backcrossed to determine if the transgene has been incorporated into the germ cells of the mice and therefore can be passed on to create a knock out line of mice. The creation of knock out mice is useful in determining the role of genes in development, or for instance in tumour formation as the effect of the loss of the gene can be examined (Loadish 2000). The *Cre-lox* system has also been used to create knock out mice. *Lox*P sites are sites of DNA recombination and *Cre* is an enzyme that can catalyse recombination between these sites. *Lox*P sites are placed to flank the gene of interest in one mouse, and a *Cre* transgene under the control of a specific promoter is introduced into another mouse. When both of these transgenic mice are mated the *Cre* will induce recombination between the *lox*P sites disrupting the target gene at the sites of Cre expression (Loadish 2000).

4.1.2 Reporter genes

So-called reporter genes can be used for ready detection of transgenic activity. These reporter genes are easy to assay against endogenous gene expression and can be detected quantitatively or qualitatively. Two commonly used reporter genes are *LacZ* and *luciferase.* The *lacZ* gene codes for β-galactosidase, which breaks down lactose. It also has the ability to break down X-Gal (5-bromo-4-chloro-indolyl-β, D-galactosidase) to 5-bromo-4-chlorindigo, which is detectable as it is blue in colour. This allows transgene expression patterns to be visualised and the expression can also be measured using a colorimeter. However this is not the most sensitive system available for quantative analysis. Another reporter gene is *luciferase* which uses luciferin as substrate and in the presence of ATP emits light that can be used as a measurement of transgene expression. Luciferin is not only quantifiable it is inexpensive and does not require the use of radioisotopes for detection. It can also be used to visualise expression patterns but requires luminescence sensitive imaging equipment, which is more expensive than the LacZ system and does not provide information at such high resolution.

4.1.3 Transient transfections

The process in which exogenous DNA is introduced into cells is called transfection. Various methods of gene transfer are used for the purposes of expressing a protein. It is useful for studying biochemical feature of proteins, antisense inhibition of protein expression, transcription-regulation of regions of genes using promoter-reporter fusions and the function of non-coding RNA. The advantages of transient transfection are that there is usually a high level of gene expression in the cell and it is quick and easy. Some disadvantages are that the technique requires a large amount of plasmid DNA and there is high variability in the numbers of cells taking up the exogenous DNA (Phillips 1999).

Transient transfections were carried out in this study using the luciferase reporter gene (Section 4.1.2). *In vitro* experiments are useful for cheaper and less complicated analysis of genes in particular cell lines, but do not give as comprehensive a result as *in vivo* studies. In this case transient transfections were used as a comparison with a transgenic mouse study. The major difference between using transgenic and transient transfections is that transgenics give results in a whole organism (where ever the promoter is expressed), whereas tissue culture can only give results in the cell line used, which normally are only one cell type, under more artificial conditions. An advantage is that where a construct may cause embryonic lethality in a mouse, in a cell line it may not cause

any cell damage if harvested after 24-48 hours and so some analysis can be carried out. The same luciferase reporter gene technology was used to assay results as was used in the transgenic mice.

The luciferase reporter gene was also used in experiments analysing DMR2 (Murrell 2001). Constructs using luciferase and various elements of *Igf2* were made to determine the minimum sequences required to increase expression in a methylation dependent manner. Methylation of Hha1 sites reduced expression 5-fold from the P3 promoter suggesting that the methylated region of *Igf2* DMR2 is required for maximal *Igf2* expression.

4.1.4 Previous analysis of *Igf2* and *H19*

Experiments utilising *LacZ* have been carried out on transgenic mice in which the Igf2 coding region was interrupted by *LacZ* within a 130kb YAC (yeast artificial chromosome) construct, also containing the *H19* gene in order to examine paternal and maternal differences in transmission of *Igf2* and *H19* in the embryos at E13 (Ainscough 1997). This 130kb YAC was shown to contain the elements necessary to visualise appropriate mesodermal and endodermal expression of *Igf2*. This YAC was introduced to mice lacking endogenous *H19* and at e14.5, expression was seen in the liver and skeletal muscle, especially in the tongue, and in the epithelial linings of the gut, kidneys and lungs. Expression was not seen in the cardiac and smooth muscle cells indicating that elements outside the YAC were required to emulate endogenous *H19* expression normally seen in these cells (Ainscough 2000). When the extra-embryonic tissues were examined expression was seen in the yolk sac but not in the placenta indicating that the placental *Igf2* enhancers lie beyond the region covered by the YAC (Ainscough 2000).

Previously luciferase expressing transgenic mice were used to quantify the expression for *Igf2* regulating elements in embryos and one-day-old mice (Ward 1997). *Igf2*, the P3 promoter and the luciferase gene were joined together in a construct and injected into embryos and the luciferase levels were measured in the liver and the brain. The results showed that DMR1 has an inhibitory effect on *Igf2* expression in the liver, the DMD enhances *Igf2* expression in the brain and the H19 promoter inhibits *Igf2* expression in the brain (Dell 1997). In other experiments constructs contained a combination of the *H19* promoter, *H19* enhancer, DMR1 and the CCD combined with luciferase and the P3 promoter. The expression levels of *Igf2* in the placenta and the yolk sac was examined to

investigate the link between down regulation of *Igf2* after birth and the glucocorticoid surge present at the same stage (Dell 1997).

4.1.5 Aims:

The purpose was to examine the effect of DMR1 had on *Igf2* expression and this was examined in three ways: firstly, transgenic mice expressing the luciferase transgene were examined to see expression levels in various organs. The constructs had different elements from the *Igf2-H19* gene region present to analyse the effects each element had on transgene expression. Luciferase was measured in selected organs to determine relative levels of expression. A comparison was made between male and female transmission of the transgenes in order to examine any potential expression level differences that might be the result of genomic imprinting. Also, methylation patterns associated with the transgene were analysed in these same organs to determine if there was any correlation between transgene expression from male and female inherited transgenes and the subsequent methylation patterns observed from these mice. Expressions from the different constructs were then compared in different lines of mice to see whether the presence of DMR1 increased or decreased transgene expression.

The second set of experiments carried out was the creation and examination of *LacZ* expressing transgenic mice. Constructs with or without DMR1 were injected into fertilised embryos and the mice subsequently stained to visualise transgene expression. A comparison was made between these mice to see the effect DMR1 had on expression patterns and any differences or similarities in expression were noted.

Thirdly, tissue culture cells were transfected with luciferase expressing constructs and the expression levels were measured and comparisons were made between the different constructs. The constructs were then methylated to analyse the effect that methylation had on expression from constructs containing DMR1. Any correlation between *In vivo* (transgenic mice) and *In vitro* (transient transfections) luciferase experiments were noted.

4.2.0 Results:

Reporter gene constructs used in transgenic mice and in transient transfection experiments are shown in Figure 1.

4.2.1 Luciferase expression in mice carrying luciferase transgenes

4.2.1.1 Transgenic expression in the Eva and Elvis lines at e14.5

In the Eva line of mice luciferase expression levels were highest in the body and lowest in the head (Figure 2). In Elvis mice however luciferase expression was highest in the head and lowest in the yolk sac (Figure 3). In both lines there was higher average expression in the embryo proper than in the extra embryonic tissues. In the comparison between male and female transgene transmission in the mice expressing construct E no statistically significant difference was found between the sexes.

4.2.1.2 Transgenic expression in the Titus, Tilly and Tracy lines at e14.5

In the Titus pups highest transgene expression was observed in the body and lowest expression was observed in the yolk sac (Figure 4). In Tilly litters highest expression was again seen in the body but high levels were also observed in the yolk sac (Figure 5). This is also true in Tracy mice where both the head and yolk sac have highest expression of the transgene (Figure 6).

Comparisons of transgene expression following either maternal or paternal transmission of the transgene were carried out in the Titus and Tilly lines and in each case no statistically significant differences were found.

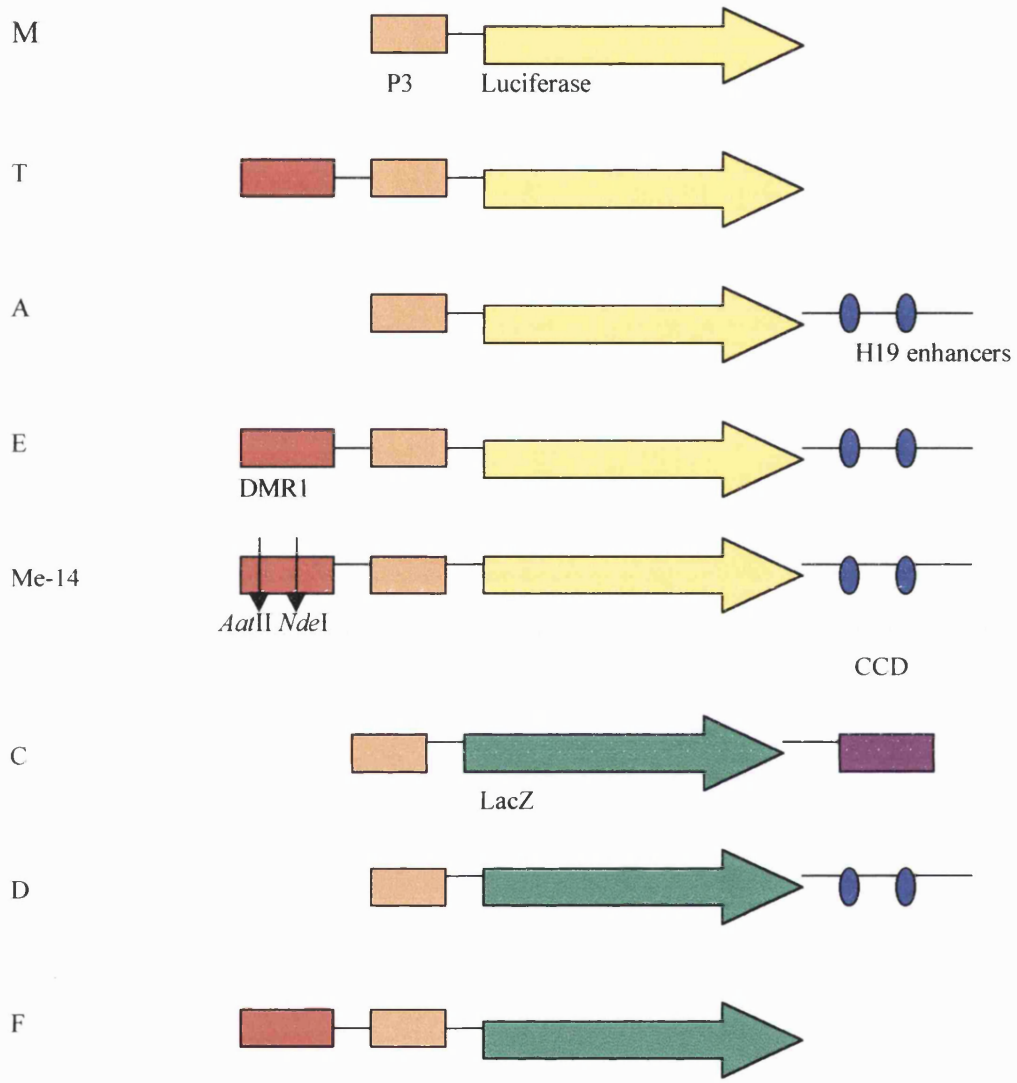


Figure 1. Constructs used in transient transfections and transgenics experiments.
The first column of letters and numbers represents construct name. The regions represented are: Differentially methylated region 1 (DMR1 red boxes), the *Igf2* P3 promoter (orange boxes), luciferase reporter gene (yellow arrow), lacZ reporter gene (green arrow), position of H19 enhancers (blue circles) and the centrally conserved domain (CCD, purple box). Black vertical arrows denote the restriction sites *Aat*II and *Nde*I.

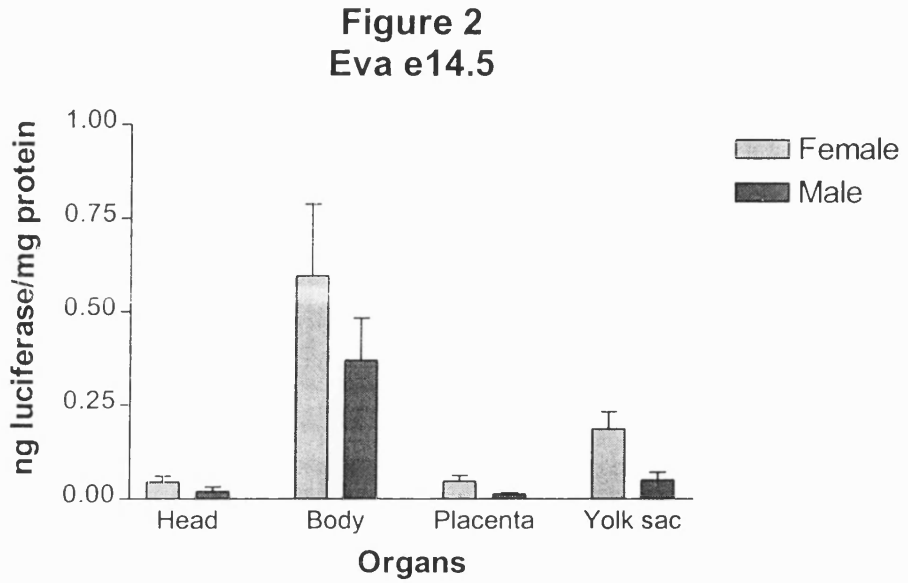


Figure 2: Luciferase levels of organ homogenates following either male or female transmission of the transgene in Eva mice at e14.5.

Organs represent parts of embryos analysed, and male and female columns represent male and female transmission of the transgene. Results of Mann-Whitney analysis: Head P= 0.2 n/s, Body P= 0.7 n/s, Placenta P= 0.1 n/s, Yolk sac P= 0.7 n/s.

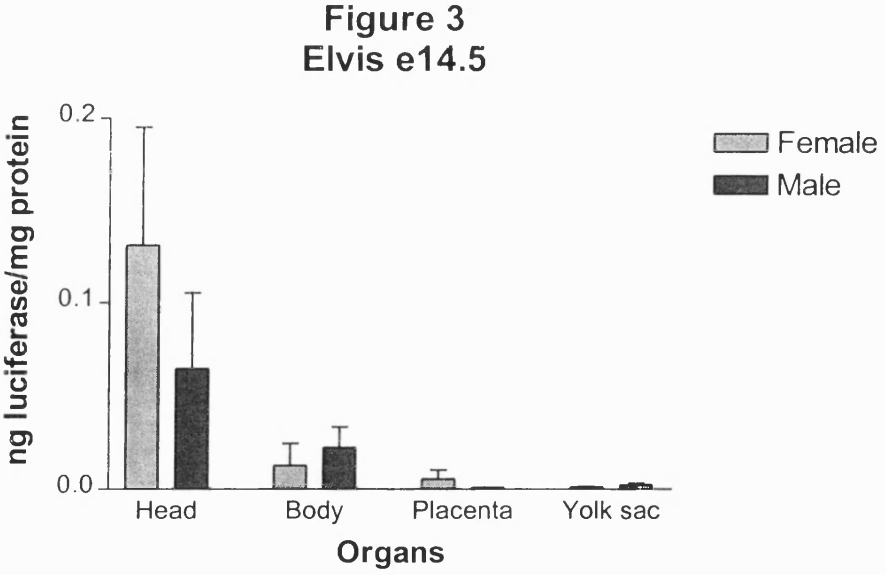


Figure 3: Luciferase levels of organ homogenates following either male or female transmission of the transgene in Elvis e14.5 mice.

Organs represent parts of the embryo analysed and male and female columns represent male and female transmission of the transgene. Mann-Whitney analysis results: Head P= 1.0 n/s, Body P= 0.0057 n/s, Placenta P= 1.0 n/s, Yolk sac P= 0.25 n/s.

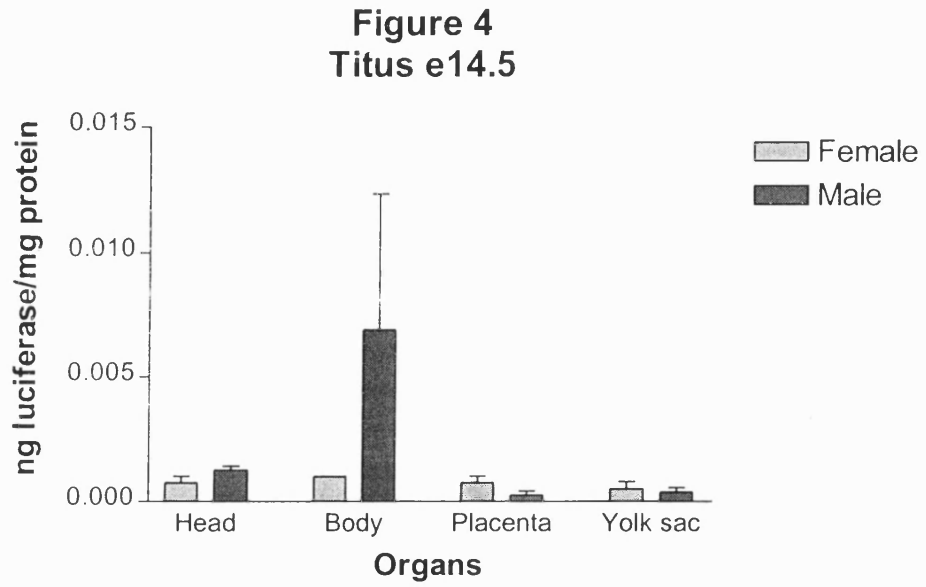


Figure 4: Luciferase levels of organ homogenates following both male and female transmission of the transgene in Titus e14.5 mice.

Organs represent parts of the embryos analysed and male and female columns represent male and female transmission of the transgene. Mann-Whitney results: Head P=1 n/s, Body P=0.23 n/s, Placenta P= 0.4 n/s, Yolk sac Not enough samples.n=4

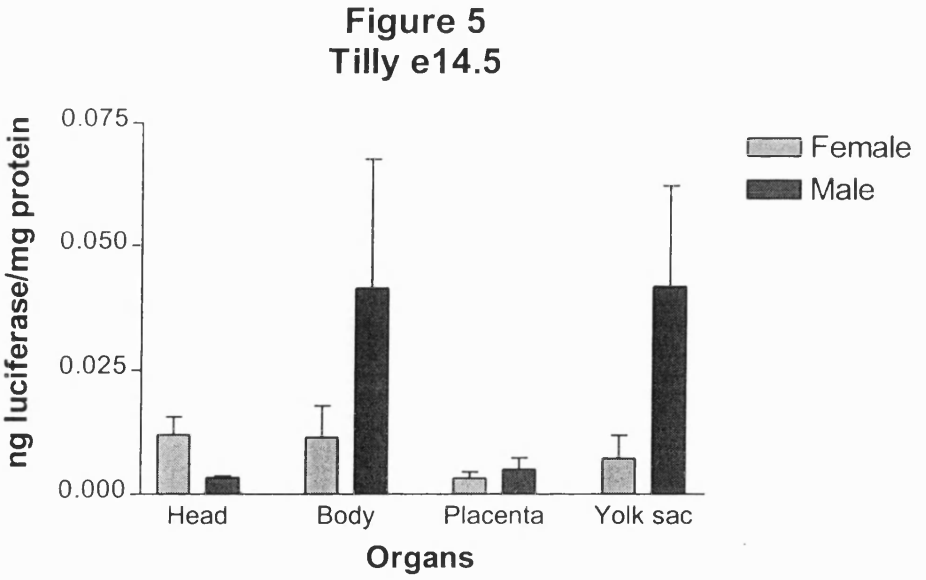


Figure 5: Luciferase levels of organ homogenates following both male and female transmission of the transgene in Tilly e14.5 mice.

Organs represent parts of the embryos analysed and male and female columns represent male and female transmission of the transgene. Mann-Whitney results: Head P= 0.057 n/s, Body P= 0.4 n/s, Placenta P= 0.857, Yolk sac P= 0.4 n/s. n=4

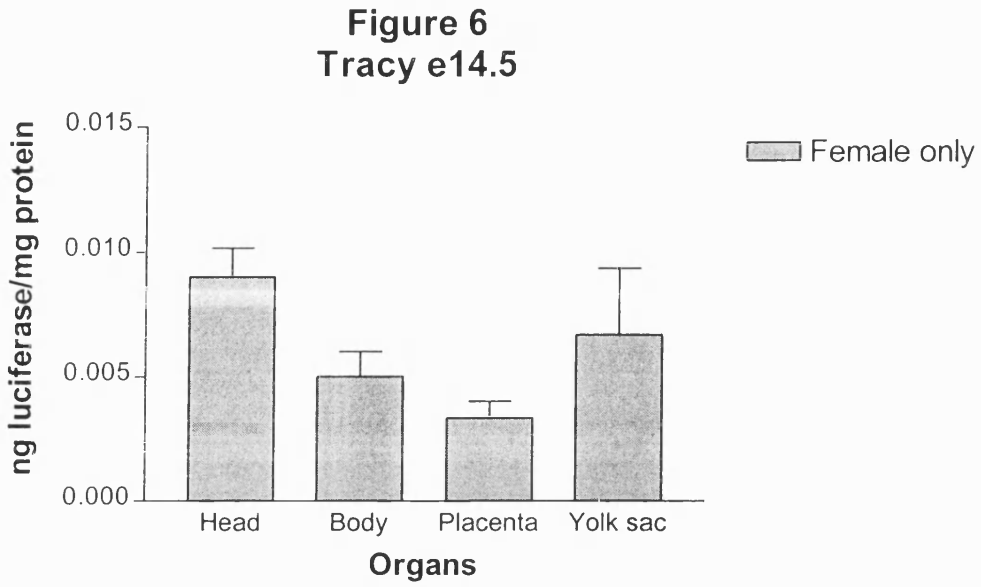


Figure 6: Luciferase levels of organ homogenates following female transmission of the transgene in Tracy e14.5 mice.
Organs represent parts of the embryos analysed female columns represent female transmission of the transgene. n=3

4.2.1. Transgene expression of T and E constructs at e14.5

Statistical analysis of the expression levels of all the T lines (Titus, Tilly and Tracy) compared with all the E lines (Eva and Elvis) showed significant differences in expression levels in the head and the body but no differences in the placenta and the yolk sac (Figure 7).

Figure 7

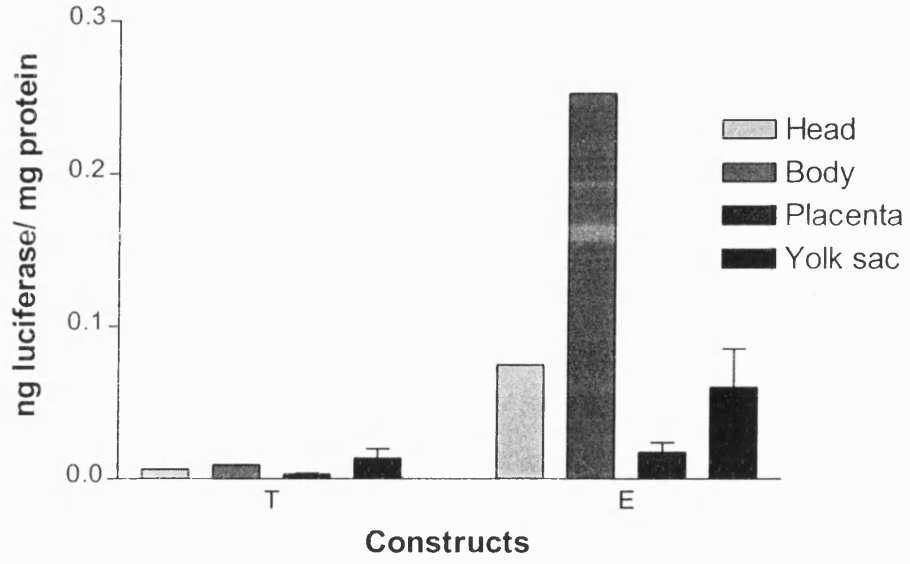


Figure 7: graph representing luciferase levels of organ homogenates of both T and E expressing mouse lines at e14.5.

Constructs represent which luciferase construct the mice are expressing and columns represent body parts analysed. Mann-Whitney results of T vs. E: Head P= 0.0021 is significant. Body P= 0.012, Is significant. Placenta P= 0.218, n/sYolk sac P= 0.326 n/s.

4. 2.1.4 Transgene expression in Eva and Elvis lines at day1

Luciferase levels in the Eva and Elvis lines were measured when the mice were one day old. In the Eva mice luciferase expression was highest in the liver and lowest in the tongue. In the Elvis mice expression was highest in the tongue and lowest in the muscle. Comparisons of transgene expression following either maternal or paternal transmission of the transgene were carried out and no statistically significant differences were found. (Figure 8 and Figure 9).

4.2.1.5 Transgene expression in Titus, Tilly and Tracy lines at day1

There were no differences in transgene expression following male and female transmission of the transgenes where there were enough samples for statistical analysis to be carried out, in the Titus (Figure 10), Tracy (Figure 11) or Tilly (Figure 12) lines. Following female transmission of the transgene in the Titus mice, expression levels were highest in the muscle and lowest in the kidney and brain. Following male transmission of the transgene

expression was highest in the kidneys and lowest in muscle. In the Tracy mice highest expression following female transgene transmission was seen in the tongue and lowest expression was seen in the liver. Following male transgene transmission, highest expression was seen in the muscle and lowest expression was seen in the liver. In the Tilly mice following female transmission of the transgene, expression levels were highest in the tongue and lowest in the liver.

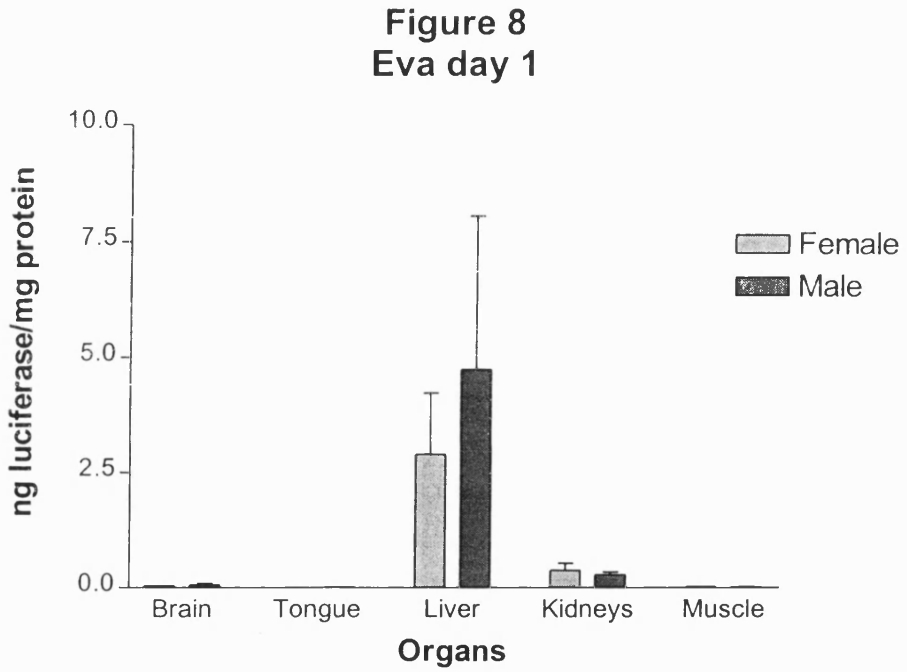


Figure 8:Luciferase levels of organ homogenates following either male or female transmission of the transgene in Eva day1 mice.

Organs represent parts of the embryos analysed and male and female columns represent male and female transmission of the transgene.

Mann-Whitney results: Brain P=1 n/s, Tongue P=4 n/s, Liver P= 1 n/s, Kidney P= 0.7 n/s, Muscle P 0.7 n/s

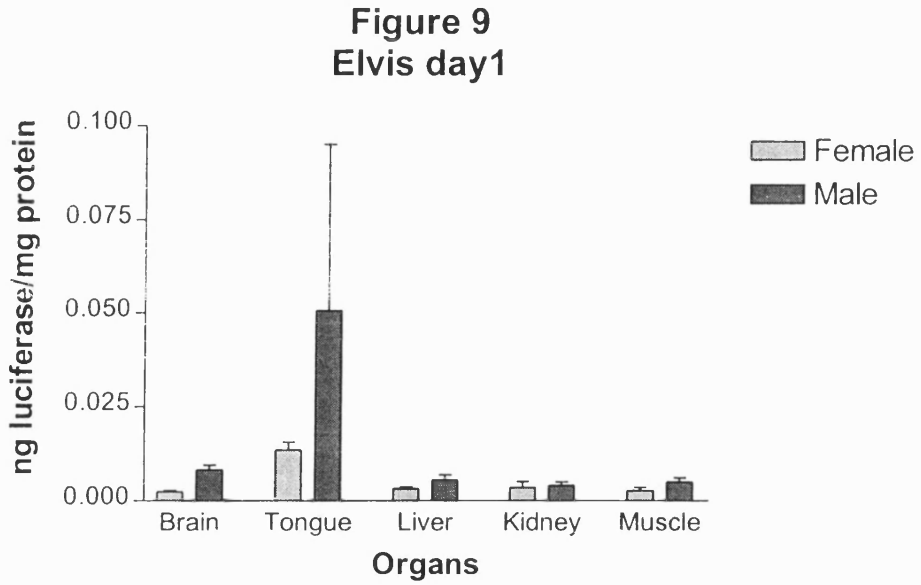


Figure 9: Luciferase levels of organ homogenates following either male or female transmission of the transgene in Elvis day1 mice.

Organs represent parts of the embryos analysed and male and female columns represent male and female transmission of the transgene. Mann-Whitney results: Brain P= 0.1 n/s, Tongue P= 0.9 n/s, Liver P= 0.23 n/s, Kidney P= 0.66 n/s, Muscle P=0.25 n/s.

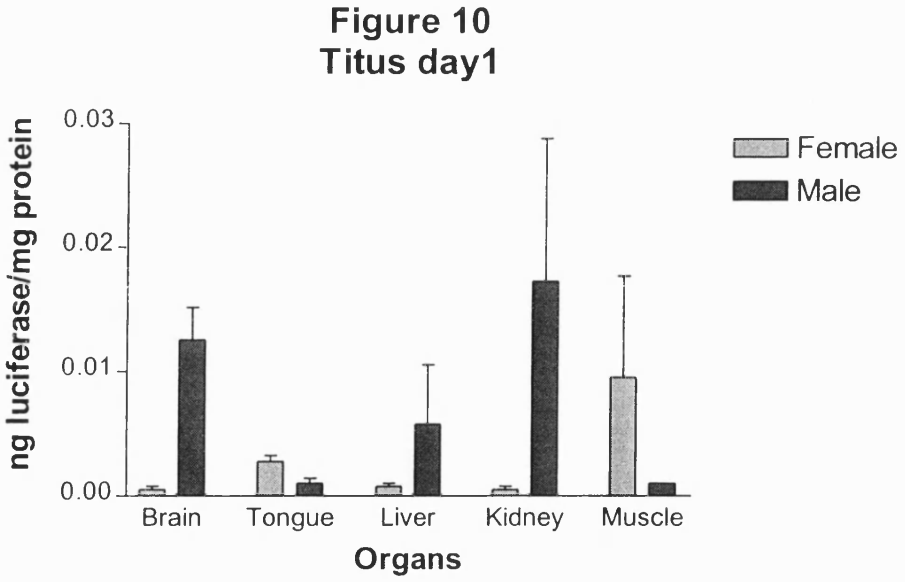


Figure 10: Luciferase levels of organ homogenates following both male and female transmission of the transgene in Titus day1 mice.

Organs represent parts of the embryos analysed and male and female columns represent male and female transmission of the transgene.

Mann-Whitney results: Brain P= 0.057 n/s, Tongue P= 0.114 n/s, Liver P= 0.342 n/s, Kidney P= 0.114 n/s, Muscle P= 0.485 n/s.

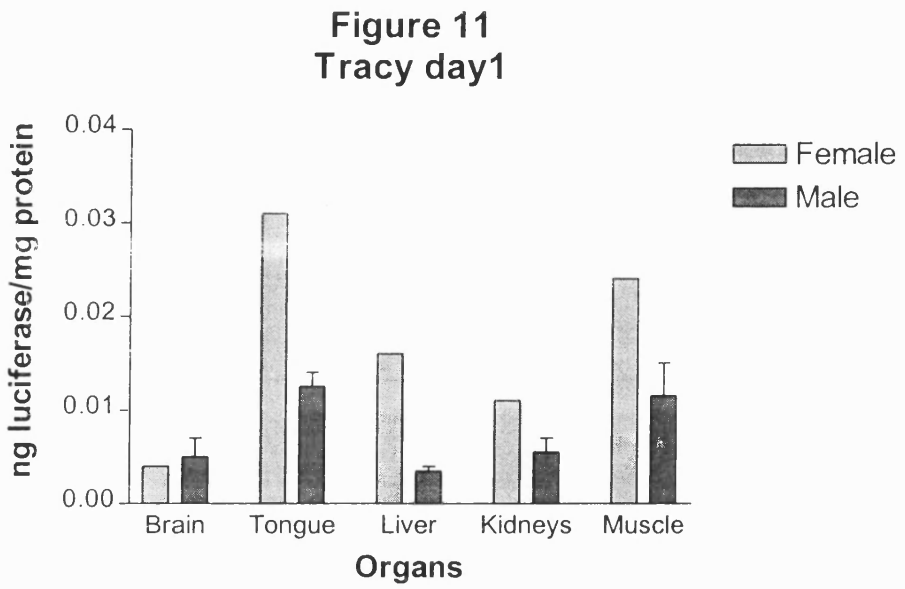


Figure 11: Luciferase levels of organ homogenates following both male and female transmission of the transgene in Tracy day1 mice.

Organs represent parts of the embryos analysed and male and female columns represent male and female transmission of the transgene. Not enough samples to perform stats. (F n=1, M N=2)

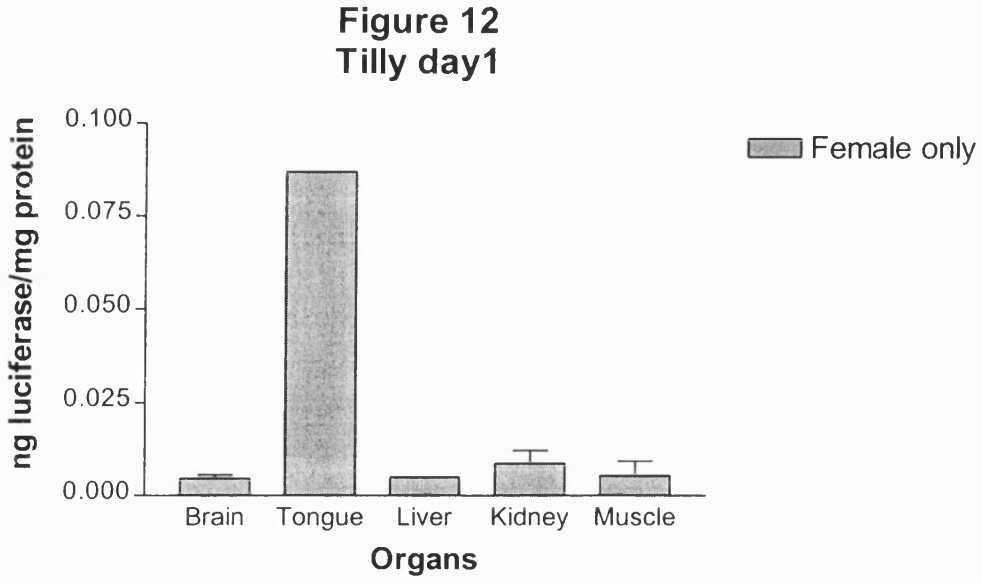


Figure 12: Luciferase levels of organ homogenates following female transmission of the transgene in Tilly day1 mice.

Organs represent parts of the embryos analysed and female columns represent female transmission of the transgene.

4.2.1.6 Transgene expression T and E lines at day1.

At day1 there is a significant difference in expression between the E and T lines of mice in the liver (Figure 13). There was no significant difference between the expression levels detected in any of the other organs analysed.

Figure 13

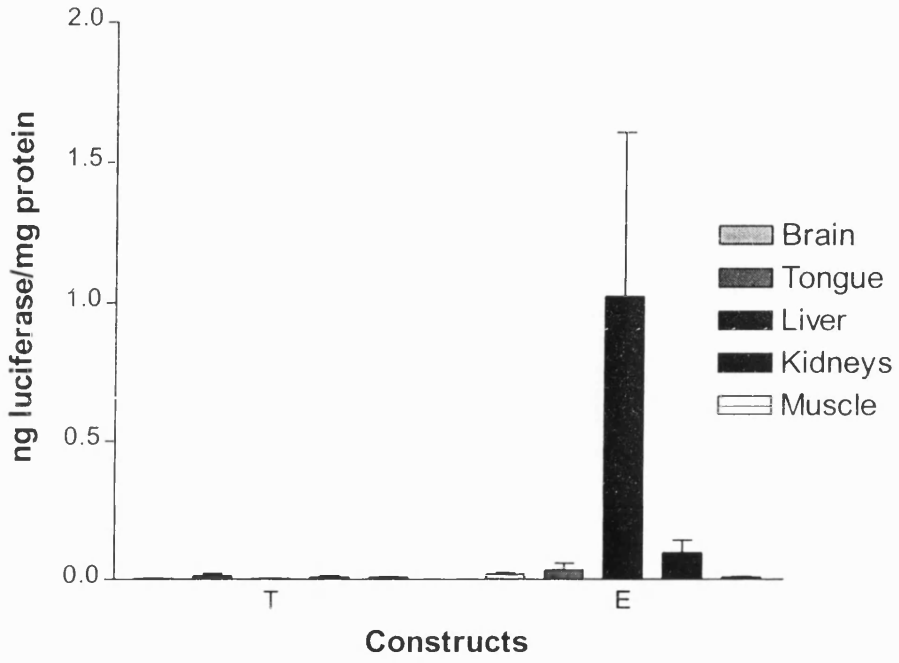


Figure 13: graph representing luciferase levels of organ homogenates of T andE expressing lines at day1.
Constructs represent constructs expressed and columns represent organs analysed.
Mann-Whitney analysis: Brain P= 0.063 n/s, Tongue P= 0.104 n/s, Liver P= 0.012 is significant. Kidneys P= 0.554 n/s, Muscle P= 0.296 n/s.

4.2.2 Male verses female transgene transmission

In the summary of analysis (Table 1) of male versus female transmission at e14.5 there was higher female transgene expression in exactly half of the organs examined. Eva always had higher expression following female transmission, Elvis had mostly higher expression following male transmission, Titus was evenly divided between the sexes and Tilly had mostly higher expression levels following male transmission of the transgene. In the head expression was mostly higher from the female transgene, in the body there was mostly higher expression following male transmission and in the placenta and yolk sac is evenly split.

	Head	Body	Placenta	Yolk sac
Eva	♀	♀	♀	♀
Elvis	♀	♂	♂	♂
Titus	♂	♂	♀	♀
Tilly	♀	♂	♂	♂

Table 1. Summary of relative levels of transgene expression following male or female transmission at e14.5.
Rows show transgenic mouse lines and columns show organs analysed. The symbols represent whether expression was higher following male (♂) or female (♀) transmission.

At day1 (Table 2) the Eva lines had mostly higher expression following male transmission, Elvis had all higher expression following male transmission, Titus had mostly higher expression following male transmission and Tracy had mostly higher expression following female transmission.

In the brain expression was always higher when transmitted from the male transgene, in the tongue, liver and kidney it was mostly higher when transmitted from the male transgene and in the muscle it was mostly higher when transmitted from the female transgene.

	Brain	Tongue	Liver	Kidney	Muscle
Eva	♂	♂	♂	♀	♀
Elvis	♂	♂	♂	♂	♂
Titus	♂	♀	♂	♂	♀
Tracy	♂	♀	♀	♀	♀

Table 2. Summary of relative levels of transgene expression following male or female transmission at day1.
Rows show transgenic mouse lines and columns show organs analysed. The symbols represent whether expression was higher following male (♂) or female (♀) transmission.

4.2.3Blots

Figure 14 shows a selection of southern blots carried out on the luciferase expressing organs. Genomic DNA was digested with *HpaII* which can only digest unmethylated DNA and also digested with *MspI* which can digest both methylated and unmethylated DNA.

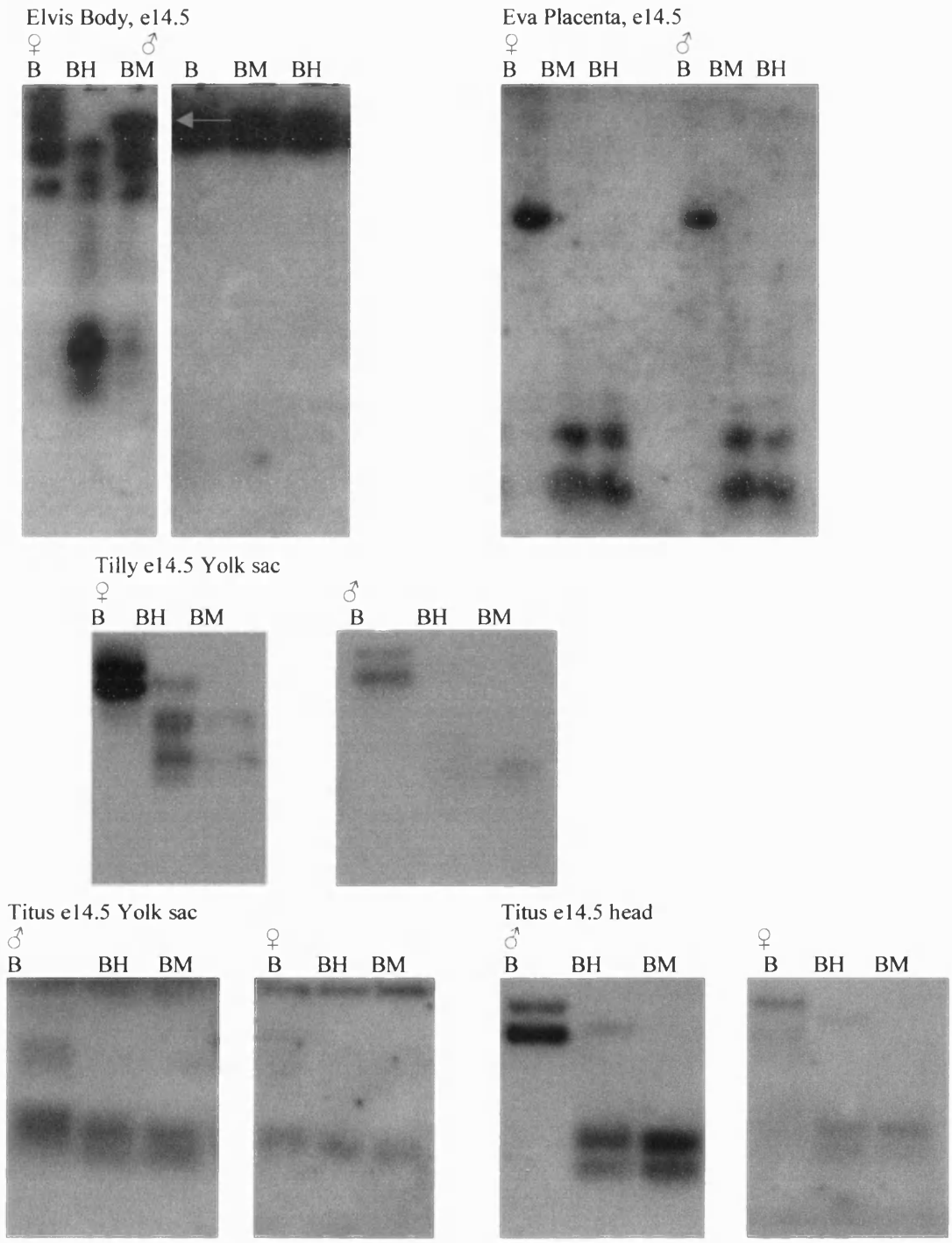


Figure 14: Sections of methylation sensitive blots of DNA from Elvis body, Eva placenta, Tilly yolk sac, Titus yolk sac and Titus head.

♂ refers to male transgene transmission; ♀ refers to female transgene transmission. B stands for *BamH1* digest, BH stands for a *BamH1* and *HpaII* digest and BM stands for a *BamH1* and *MspI* digest. Red arrow indicates differences in band patterns between equivalent digests.

4.3 LacZ transgenic mice:

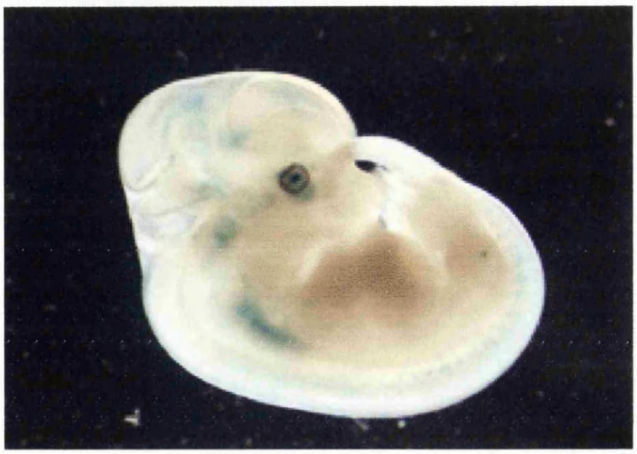
4.4.1 C Construct

The first construct injected into embryos was C (Figure 15) which was generated by M. Charalambous who also genotyped all subsequent litters and all further analysis. However construct C was really only used as a reference construct and so no further comment on the results are made in this thesis, but can be found in (Charalambous 2004). A summary of the injections carried out can be found in the appendix. Two PCR positive mice were found but only one was discovered to transmit the transgene onto the next generation (Christian). Two other C mice were created by M. Charalambous called Cornelius and Columbo. Below is a comparison of the expression patterns showed by these three transgenic mice.

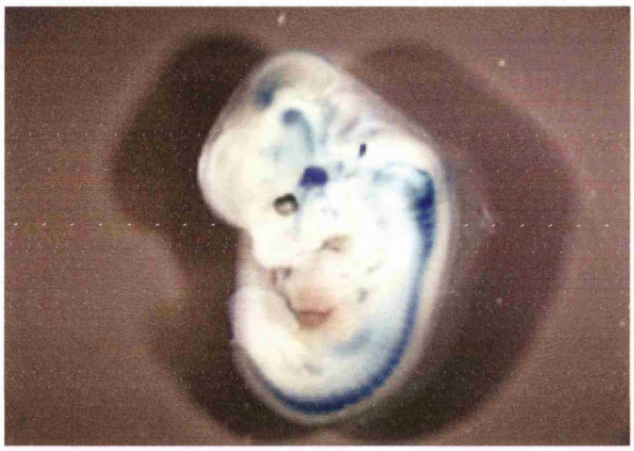
	Christian	**Columbo**	**Cornelius**
Choroid plexus	✓	✓	✓
Meninges	✓	✓	✓
Somite derivatives	X	X	X
Tongue	✓	✓	✓
Cartilage (ear and neck)	✓	✓	✓

Table 3. Summary of expression patterns of the C transgene in the Christian, Columbo and Cornelius lines.

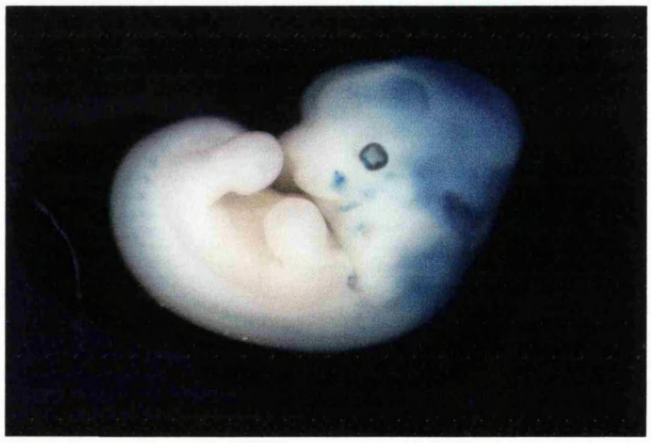
Cornelius, Christian and Columbus are transgenic mice expressing the C transgene. The ticks indicate those areas where there was expression and the crosses indicate those organs without transgene expression.



Christian



Cornelius



Columbo

Figure 15: Comparison of LacZ staining in three lines of mice expressing the C construct at e12.5. Blue areas are sites of transgene expression. The Christian line was generated by S. Kelly, and the Cornelius and Columbo lines were generated by M. Charalambous.

4.3.2 D construct:

Construct D was created by M. Charalambous and two PCR positives were found when litters were genotyped. However no transgenic positive pups were found when these PCR+ve mice (Dave and Daphne) were mated so it possible that these mice were mosaic for the transgene and did not pass it on to their offspring. A summary of the numerous injections carried out can be found in the appendix. Dilutions of the transgene were tried but with no further success

One possibility is that the construct was toxic to the embryos. Litter sizes were consistently smaller than average, which sometimes led to the pups being destroyed and ingested by the mother. This happens if the energy that would have been expended by the mother in rearing the litter would out weigh the benefits of having a small litter.

4.3.3 F constructs:

One potential founder (named Fran) was identified when genotyped as PCR positive but subsequent mating did not produce a transgenic mouse line. Like Dave and Daphne, Fran did not pass the transgene onto the next generation and so was possibly mosaic. As no transgenic mice were forthcoming, from the 24/1/02 to the 21/3/03, the pseudopregnant mice were not allowed to go to term but the embryos were removed at e14.5 and stained for *LacZ*. This resulted in two blue and therefore two transgenic mice (Figure 16). However no lines could be created, as the mice were still embryos when dissected. A summary of the injections carried out can be found in the appendix.

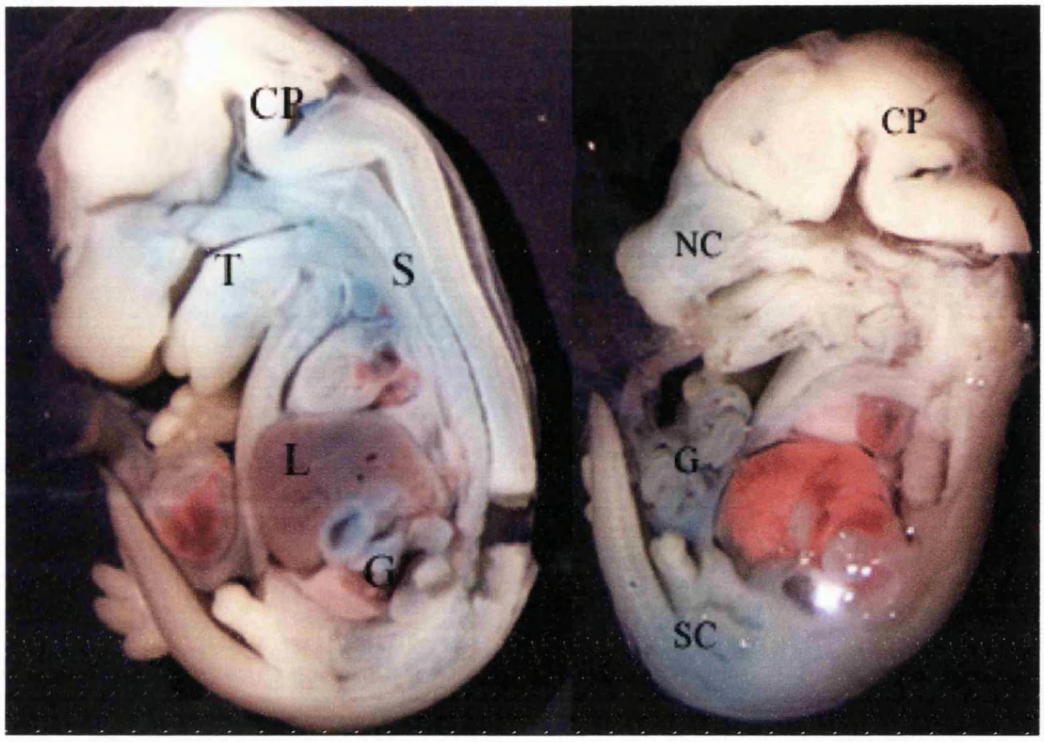


Figure16: Comparison of LacZ staining of transgenic mice Fa and Fb expressing construct F at e14.5. Blue areas show sites of transgene expression. Cp shows the choroid plexus, T the tongue, NC nasal cartilage, G the gut, L the liver, S and SC the spinal column.

4.3.4 Comparison between mice expressing construct F at e14.5

Mice expressing construct F were sectioned and examined to determine the expression pattern of the transgene.

Organs	Fa	Fb
Choroid Plexus	✓	✓
Tongue	✓	✓
Nasal cartilage	✓	✓
Mid-forebrain	✓	✓
Spinal cord	✓	✓
Meninges	✓	X
Intestine	✓	✓
Liver	✓	X

Table 4. Comparison between Fa and Fb transiently transgenic mice.
Fa and Fb are transgenic mice expressing construct F. The ticks indicate which organs express the transgene and the crosses indicate which organs did not express the transgene.

4.4 Transient transfections:

Constructs M, T, A, E and Me-14 were transfected into HepG2 cells and luciferase expression levels were assayed (Figure 17). Construct A had the highest expression level, T was the next highest closely followed by M. Constructs E and Me-14 were the lowest expressing constructs.

When these constructs were methylated in a separate series of transfections the expression levels changed (Figure 18). Luciferase levels are much lower in the second series of transfection than in the first series. Also, the unmethylated constructs were expected to behave as the unmethylated constructs in the first series of transfections, which they did not. Construct A did not have the highest luciferase level as it did initially, and which was the expected result. When methylated, construct Me-14 was the most highly expressed and construct A had the lowest expression level with statistical analysis showing no significant differences between methylated and unmethylated constructs.

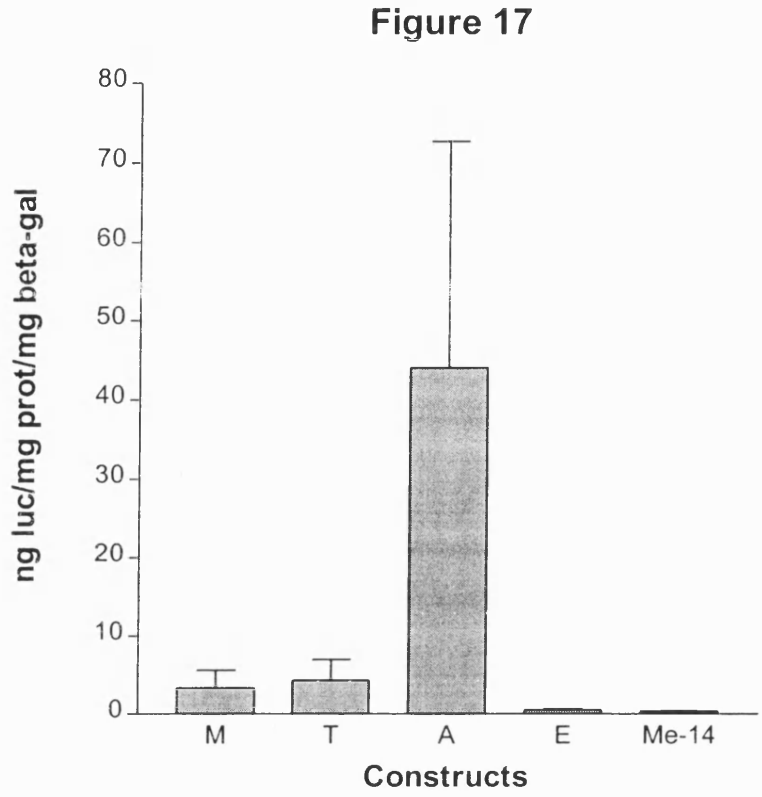


Figure 17. Luciferase levels of constructs transfected into HepG2 cells.
The letters on the X-axis represent the constructs transfected into the HepG2 cells. M has P3 promoter and the luciferase reporter gene, T had DMR1, P3 and luciferase, A had P3, Luciferase and the H19 enhancers, E had DMR1, P3, Luciferase and the H19 enhancers and finally Me-14 had DMR1 with two added restriction sites (*AatII, NdeI*), P3, luciferase and the H19 enhancers.

Figure 18

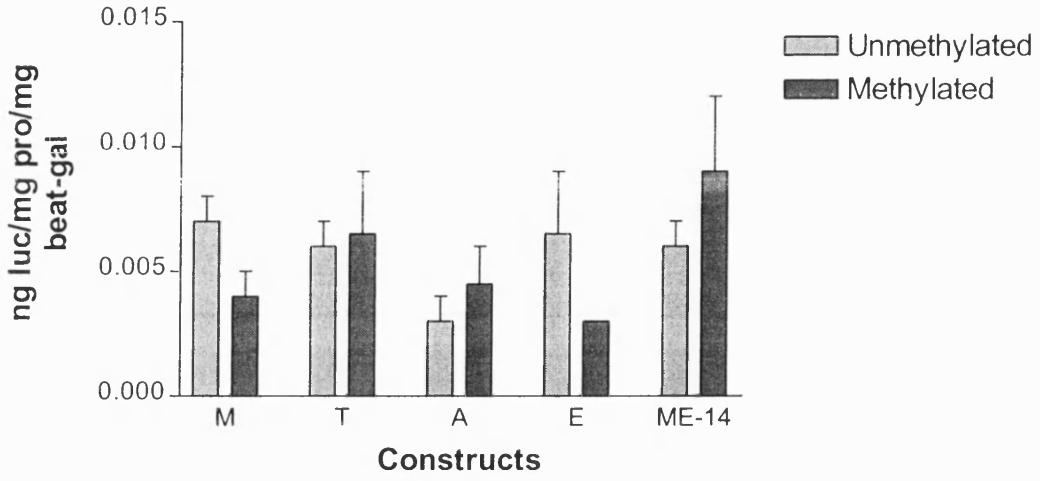


Figure 18. Luciferase levels of unmethylated and methylated constructed transfected into HepG2 cells. The letters on the X-axis represent the constructs transfected into the HepG2 cells. M has P3 promoter and the luciferase reporter gene, T had DMR1, P3 and luciferase, A had P3, Luciferase and the H19 enhancers, E had DMR1, P3, Luciferase and the H19 enhancers and finally Me-14 had DMR1 with two added restriction sites (*AatII, NdeI*), P3, luciferase and the H19 enhancers.

Discussion:

Chapter Four: Analysis of DMR1 in transgenic mice and in transient transfections.

Analysis of transgenic mice expressing luciferase reporter gene and injection of embryos of mice with LacZ reporter gene to analyse elements of Igf2/H19 gene region.

4.0 Analysis of Eva and Elvis transgenic mice.

When assayed Eva and Elvis lines were compared at e14.5 there were differences in relative expression levels in the organs. Eva had highest expression in the body and Elvis had highest expression in the head. Even though both lines were injected with the same construct the transgene enters the genome in random positions and the differences in expression levels between Eva and Elvis could be due to positional effects or due to transgene copy number. The genomic elements surrounding the site of transgene integration may have inhibitory effects on the luciferase gene. Both lines have higher expression in the embryo than in the extra embryonic tissues. This implies that DMR1, P3 and the H19 enhancers are more active in the embryo than in the yolk sac and placenta. H19 enhancers are known to increase transgene expression levels 10-20-fold in the brain (Ward 1997), which could explain the higher transmission in the embryo than detected in the extra embryonic tissues.

In the day1 samples expression was highest in the liver in Eva and highest in the tongue in Elvis mice. This correlates with the results found at e14.5. The transgene shows increased expression in the body at e14.5 of Eva mice and the liver at day1. It is possible that in the body homogenates it is primarily the liver that increases expression levels and this becomes apparent in the more detailed dissections carried out at day 1. This could also be true for the Elvis mice with the expression in the tongue being responsible for the high expression levels found in the head homogenates at e14.5. The enhancer activity of H19 enhancers in the liver was demonstrated previously (Ward 1997) with the addition of H19 enhancers increasing transgene expression many thousand fold when E and T lines were compared. This was maintained in the liver up to 4 days postnatally.

Methylation sensitive blots carried out on samples from Elvis and Eva mice showed methylation differences in Elvis e14.5 ♀ body between lane two and three. There was an extra band in the *MspI* lane where the enzyme digested the DNA where the *HpaII* could not due to methylation. This methylation occurred in the maternally transgene transmitted sample and not in the paternally transmitted sample, but as shown in Figure 3. there was higher transmission from the paternally expressed transgene. It appears that the methylation of the maternally expressed transgene down regulated expression but not to a statistically

significant extent. There were no methylation differences in the Eva e14.5 blots, which correlated with there being no differences in luciferase expression following male and female transmission of the transgene.

4.1 Analysis of Titus, Tilly and Tracy transgenic mice

In the three T lines at e14.5 the highest luciferase expression was observed in the body of Titus mice, body and yolk sac of Tilly mice and head and yolk sac of Tracy mice. Construct T has DMR1, P3 and the luciferase reporter gene. In most of the T mice, both the embryo and the extra embryonic tissue have high luciferase levels. It could be the case that the H19 enhancers present in the E lines up-regulated expression in the embryo but not in the extra-embryonic tissues so that in the T lines expression levels between the embryo and the extra embryonic tissues are comparable. Also expression levels are much higher in the E lines than in the T lines, consistent with the presence of the H19 enhancers in the E transgene.

At day1 it was in the kidney that expression was highest in Titus mice, and in the tongue, expression was lowest. This agrees with what was observed at e14.5 with the more detailed dissection carried out at day one revealing the kidney as one possible source of high expression in the body homogenate. In Tracy mice expression was highest in the tongue and lowest in the brain, which again agrees with the data at e14.5, with tongue contributing to high transgene expression in the head. In Tilly mice, expression was highest in the tongue and lowest in the brain. This does not correlate with the observation at e14.5 as the liver, kidney and muscle had quite low levels of expression individually yet the body homogenate at e14.5 showed much higher expression levels. This could be due to a different organ in the body region having very high luciferase expression. Previously it was shown that DMR1 decreased transgene activity in the liver and brain, which correlates with findings in this study (Ward 1997).

The results of the methylation sensitive blots showed no methylation differences in the Tilly e14.5 yolk sacs, Titus e14.5 yolk sacs and Titus e14.5 heads.

4.2 Comparison between construct T and construct E.

The E lines had higher expression than the T lines at e14.5 which is not surprising given that both transgene constructs contain DMR1 and Igf2 P3, but construct E also contains the *H19* enhancers which are known to increase expression levels of *Igf2*.

It appears that expression is significantly higher in the embryo than it is in the extra embryonic tissues at e14.5 in the Eva and Elvis mice so it appears that the addition of *H19* enhancers does not effect expression of *Igf2* in the placenta and yolk sac.

This result also occurred at day1 with E mice having significantly higher levels of expression than T mice, but only in the liver. Expression levels were similar in all of the other organs. This correlates with previous findings (Ward 1997) that showed high expression from construct E in the liver and that the *H19* enhancers are mostly active in endodermally derived tissues.

4.3 Comparison between male and female transmission of the transgenes

There were no statistically significant differences, in any comparison made following male and female transmission of the transgene.

In Eva e14.5 pups however expression was always highest following female transmission but at day one this fell to only being highest in the kidney and muscle. It may have been expected that luciferase levels would be highest following male transmission of the transgene due to paternal methylation of DMR1, leading to up-regulation of the transgene, as occurs in the paternal allele of endogenous *Igf2*. The Elvis mice followed this expectation more closely with higher expression occurring in all organs except for the head at e14.5 following paternal transmission of the transgene.

Similarly, the Titus mice had higher expression following paternal transgene transmission in 5 out of the 9 organs analysed. Luciferase levels were generally very low in the T lines, however in Tilly mice at e14.5 there was mostly higher expression from the paternally inherited transgene except for in the head, but in the Tracy day 1 sample there was mostly higher expression levels following maternal transmission of the transgene. Liver and brain analysis carried out on transgenic mice with paternal transmission of the transgene showed high expression from E lines of mice and low expression from T lines of mice (Ward 1997). When paternal and maternal transmission of the transgene was compared, E lines of mice showed higher expression levels when paternally expressed and T lines of mice showed higher expression levels when maternally expressed.

5.0 Analysis of LacZ transgenic mice

5.1 Transgenic mice expressing construct C

One C mouse was created which was named Christian. When compared with the C mice created by M. Charalambous the expression patterns were found to be similar. When compared to each other (Charalambous 2004), common sites of expression were the leptomeninges, choroid plexus, lens of the eye, tongue, mesenchyme of the head, tubules of the ear and the ureter. Differences between the three C lines were seen in the abdominal wall muscle where only Colombo mice showed expression, facial ganglia, dorsal root ganglia and peripheral (to the mesonephros) ganglia where only Cornelius mice showed expression, and in the digital cartilage and dermis of the skin where only Christian mice showed transgene expression. The sites of differential transgene expression could be due to positional effects as the transgene probably inserted into different regions of the mouse genome in each of the three C lines.

5.2 Transgenic mice expressing construct F

Two F e14.5 transgenic mice were also created which also had similar patterns of expression as each other. Sites of common expression at e14.5 between mouse Fa and mouse Fb were the choroid plexus, nasal cartilage, rib bones, mid-forebrain, spinal column and the intestines. Sites of differential expression were the tongue muscle where only mouse Fa showed transgene expression, the meninges surrounding the spinal column where only mouse Fa showed transgene expression, the heart where there is only visible expression from mouse Fa and the liver where only mouse Fa showed transgene expression. All of these regions are derived from the mesoderm and endoderm, which means that it is possible that in mouse Fb the transgene inserted near to a meso/endodermal silencer.

5.3 Comparison of C transgenic mice with F transgenic mice.

When compared to each other the C and F lines have common sites of expression such as the choroid plexus, tongue (except for Fb), some cartilage and the meninges (except for Fb). Differences in sites of expression can be seen in the lens of the eye where there is no F transgene expression visible and in the intestines where there appears to be little or no C transgene expression. The difference between construct C and construct F is that construct C had the CCD as well as P3 and LacZ reporter gene and construct F has DMR1 as well as P3 and LacZ reporter gene. The presence of CCD leads to transgene expression in more areas of the brain than shown by construct F and to less expression in the abdomen and the organs within.

The presence of DMR1 on construct F leads to more mesodermal and endodermal organs expressing the transgene. It is thought that the CCD acts as an enhancer for Igf2 expression in the brain (Ainscough 2000) where *Igf2* is biallelically expressed and that DMR1 exerts control over *Igf2* expression in mesodermal tissues as a maternal deletion of DMR1 leads to biallelic expression of *Igf2* in mesodermal tissues (Constancia 2000).
The differences in transgene expression between the two lines of mice agree with previous findings regarding the roles of CCD and DMR1 in controlling *Igf2* expression.

6.0 Transient transfections

The results of transient transfection carried out in HepG2 cells are shown in Figure 17. Construct A had the highest expression levels which agrees with transgenic analysis previous carried out (Ward 1997) which showed that transgenic mice expressing construct A had the highest levels of expression. This is due to the construct containing H19 enhancers and not experiencing the silencer activity of DMR1. Construct T had the next highest level of expression which correlates with previous work where construct T when expressed in the kidney showed lower expression than A and higher expression than M, which is also true in this study. Constructs E and Me-14 had the lowest expression levels despite the presence of H19 enhancers showing an inhibitory effect of DMR1.

These constructs were methylated and the result was a reversal in expression levels with construct A now having the lowest expression level and Me-14 having the highest. Construct A does not contain DMR1 and construct Me-14 does contain DMR1 which as previously shown, methylated DMR1 up regulates gene expression (Li, Beard et al. 1993). Expression levels of construct T (DMR1 present) remained higher than construct M (no DMR1 present). Construct E would have been expected to demonstrate the same increase in expression as construct Me-14, but expression from construct E was lowest of all the methylated constructs. However a larger sample size may give a more conclusive result regarding this construct.

5.0 Conclusions

In Chapter three the aim was to determine if:

1. Deletion of DMR1 resulted in a different tumour spectrum than had been observed in previous experiments carried out on p53 knockout mice?

The tumour spectrum in this study was not significantly different from previous observations. These DMR1 knockout/p53knockout mice typically showed tumours of the thymus, lymph nodes and spleen which aggress with mice analysed which were p53knockout only.

2. Do tumours develop sooner or later than had been previously observed?

Tumours did not occur sooner or later in DMR1knockout/p53 knockout mice than occurred in p53knockout mice. Altering DMR1 status did not result in a shorter or longer life span for these mice and tumour development did not occur earlier or later in development.

3. Does a deletion of DMR1 result in heavier or lighter mice?

Deletion of DMR1 results in prolonged postnatal expression of *Igf2*. It appears that at 4 weeks wtDMR1 mice were the heaviest with the prolonged higher levels of circulating *Igf2* not resulting in heavier mice. However at 8 weeks, prolonged *Igf2* expression in paternal deletions of DMR1 resulted in heavier than wild type mice. This result had disappeared at 9-10 months of ages indicating that Igf2 levels had reverted to normal levels.
Wild type mice were shown to have the heaviest organs, the highest percentages of body fat, the steepest growth curves and the most visible glycogen in the liver sections.

The aims of chapter four were to determine if:

4. Analysis of the function of elements present on *H19/Igf2* gene region both *In vitro* and *In vivo?*

In transgenic mice expressing constructs with *H19* enhancers, expression is upregulated in both extra embryonic and embryonic tissues, but expression is significantly higher in the embryo than in the placenta and yolk sac. *H19* enhancers have a less upregulatory effect in the extra embryonic tissues.

5. Were there any differences between male and female transmission of transgenes?

There were no significant differences between male and female transmission of trangenes which is not surprising considering none of the construct contained the ICR which sets the imprint for this gene region and without it imprinting marks do not follow endogenous patterns of expression. Also there were no differences in methylation in these mice.

6. In which organs was expression found to be the highest?

In the liver, luciferase expression was found to be highest when the *H19* enhancers were present. Where DMR1 was present exclusively expression was generally highest in the tongue.

Appendix

1.1 Injections carried out with transgene C.

Table 1. summarises the injections carried out with construct C over a period of four months. Table 2. summarises injections carried out over a period of 2 years using transgene construct D. On the 14/6/00 to the 1/6/00 the embryonic transfers were carried out by me but resulted in no pups being born. Also different concentrations of the transgene were injected on the 18/7/02, 25/7/02 and 22/8/02. These lower concentrations did not yield transgenic mice. Table 3 summarises injections carried out over a period of 2 years using construct F. From the 28/6/01 to the 20/12/01 half of the embryonic transfers were carried out by A. Ward and half by S. Kelly. From the 24/1/02 to the 21/3/03 the pseudopregnant mice were not allowed to go to term but the embryos were removed at E14.5 and stained for LacZ. This resulted in two blue and therefore two transgenic mice. However no lines could be created, as the mice were still embryos when dissected.

Injection date	No. injected	DOB	No. born	Analysis
12/4/00	25 embryos	1/5/00	4	All –ve
15/5/00	28 embryos	29/5/00	3	All –ve
24/5/00	56 embryos	13/6/00	10	All –ve
31/5/00	28 embryos	None born	0	-----
6/7/00	60 embryos	25/7/00	10	1 PCR +ve
19/7/00	37 embryos	None born	0	-----
17/8/00	50 embryos	5/9/00	8	All –ve
31/8/00	45 embryos	19/9/00	7	1 PCR +ve

Table 1. Summary of injections carried out with transgene construct C.
The injection date refers to the date the embryos were injected with construct C and the No. Injected refers to how many embryos were injected. DOB indicates the date of birth of any subsequent pups and No. born indicates how many pups were in that litter. The Analysis column indicates the results when the pups were genotyped, All-ve meaning that all the pups were PCR negative, and +ve indicates how many were PCR positive for the transgene.

Injection date	No. injected	DOB	No. born	Analysis
7/9/00	45	None born	0	-----
14/9/00	43	3/10/00	7	All –ve
21/9/00	54	None born	0	-----
28/9/00	32	17/10/00	13	All –ve
5/10/00	52*	25/10/00	4	1 PCR +ve (Dave)
12/10/00	43	1/11/00	2	1 PCR+ve (Daphne)
26/10/00	32*	None born	0	-----
2/11/00	40*	None born	0	-----
16/11/00	34*	None born	0	-----
24/11/00	30	None born	0	-----
7/12/00	40*	None born	0	-----
14/12/00	39*	None born	0	-----
21/6/00	33	None born	0	-----
14/6/00	50*	4/7/02	2	All -ve
7/6/00	40*	None born	0	-----
1/6/00	50*	None born	0	-----
21/6/00	33	None born	0	-----
8/2/02	50	None born	0	-----
22/2/02	22	None born	0	-----
23/5/02	38	None born	0	-----
18/7/02	25 (1/10)	None born	0	-----
25/7/02	45 (1/10)	None born	0	-----
22/8/02	50 (1/50)	11/0/02	7	All -ve
12/9/02	25	None born	0	-----
17/10/02	30	None born	0	-----
14/11/02	32	None born	0	-----

Table 2. Summary of injections carried out with transgene construct D.
The injection date refers to the date the embryos were injected with construct D and the No. Injected refers to how many embryos were injected. DOB indicates the date of birth of any subsequent pups and No. born indicates how many pups were in that litter. The Analysis column indicates the results when the pups were genotyped, All-ve meaning that all the pups were PCR negative, and +ve indicates how many were PCR positive for the transgene. The astirex indicate when surgery was carried out by S. Kelly. The numbers in brackets indicate the dilution of the transgene injected.

Injection date	No. injected	DOB	No. born	Analysis
28/6/01	55*	17/7/01	7	1 PCR +ve(Fran)
5/7/01	66*	None born	0	-----
12/7/01	45*	31/7/01	7	All –ve
19/7/01	40*	None born	0	-----
6/9/01	55*	None born	0	-----
13/9/01	43*	None born	0	-----
20/9/01	76*	10/10/01	7	All –ve
27/9/01	25*	None born	0	-----
4/10/01	50*	None born	0	-----
11/10/01	60*	None born	0	-----
18/10/01	72*	6/11/01	3	All –ve
22/11/01	38*	10/12/01	4	All –ve
29/11/01	44 (1/3) *	None born	0	-----
7/12/01	36 (1/3)*	None born	0	-----
20/12/01	53 (1/3)*	8/1/02	7	All –ve
18/1/02	18	None born	0	-----
24/1/02	58	None born	0	-----
7/2/02	60	None born	0	-----
14/2/02	55	None born	0	-----
21/2/02	41	None born	0	-----
28/2/02	30	20/3/02	3	All -ve
14/3/02	50	3/4/02	5	All -ve
28/3/02	25	None born	0	-----
16/5/02	33	4/6/02	5	All -ve
30/5/02	50	None born	0	-----
13/6/02	47	None born	0	-----
13/12/02	33	None born	0	-----
24/1/02	30 (transient)	7/2/02 dissect	2	2 PCR +ve
30/1/02	21 (transient)	None Found	0	-----

6/2/02	50 (transient)	21/2/02 dissects	2	All –ve
20/2/02	51 (transient)	None found	0	-----
27/2/03	40 (transient)	14/3/03 dissects.	1	All –ve
12/3/03	46 (transient)	27/3/03 dissects.	3	All –ve
6/3/03	62 (transient)	21/3/03 dissects.	7	All –ve
21/3/03	30 (transient)	None found	0	-----

Table 3. Summary of injections carried out with transgene construct F.
The injection date refers to the date the embryos were injected with construct F and the No. Injected refers to how many embryos were injected. DOB indicates the date of birth of any subsequent pups and No. born indicates how many pups were in that litter. The Analysis column indicates the results when the pups were genotyped, All-ve meaning that all the pups were PCR negative, and +ve indicates how many were PCR positive for the transgene. The asterix indicate when surgery was carried out by S. Kelly. The numbers in brackets indicate the dilution of the transgene injected.

References

Ainscough, J., Dandolo, Surani (2000). "Appropriate expression of the mouse H19 gene utilises three or more distinct enhancer regions spread over more than 130kb." Development **91**: 365-368.

Ainscough, J., John, Barton, Surani. (2000). "A skeletal muscle-specific mouse *Igf2* repressor lies 40 kb downstream of the gene." Development. **127**.

Ainscough, J., Koide, Tada, Barton and Surani (1997). "Imprinting of Igf2 and H19 from a 130kb YAC transgene." Development **124**: 3621-3632.

Baek, S., Yoo, Cho, Choi, Sung, Mckeon, Lee. (2003). "p53 deficiency and defective mitotic checkpoint in proliferating T lymphocytes increase chromosomal instability through aberrant exit from mitotic arrest." Journal of Luekocyte Biology. **73**: 850-861.

Bartolomei, M. S., A. L. Webber, et al. (1993). "Epigenetic mechanisms underlying the imprinting of the mouse H19 gene." Genes Dev **7**(9): 1663-73.

Beard, L., Jaenisch. (1995). "Loss of methylation activates Xist in somatic but not in embryonic cells." Genes and Development **9**: 2325.

Bell, A., West, and Felsenfeld. (1999). "The Protein CTCF Is Required for the Enhancer Activity of Vertebrate Insulators." Cell **98**: 387-396.

Bestor, T. (1998). "Gene silencing: Methylation meets acetylation." Nature **393**: 311-312.

Birger, S., Perk, Razin (1999). "The imprinting box of the mouse Igf2r gene." Nature **397**: 84-88.

Bourcigaux, N., Gaston, V. Logie, Bertagna, Bouc, Gicquel. (2000). "High expression of cyclin E and G1 CDK and loss of function of p57kip2 are involved in proliferation of malignant sporadic adrenocortical tumors." The Journal of Clinical Endocrinology and Metabolism **85**(1): 322-330.

Cattanach, K. (1985). "Differential activity of maternally and paternally derived regions in mice." Nature **315**: 496-498.

Cerrato, F., Dean, Davies, Kagotani, Mitsuya, Okumura, Riccio, Reik (2003). "Paternal imprints can be established on the maternal Igf2-H19 locus without altering replication timing of DNA." Hum Mol Genet **12**(23): 3123-3132.

Charalambous, M., Bennett, Kelly, Dell, Ward. (2004). "An enhancer element at the Igf2/H19 locus drives gene expression in both imprinted and non-imprinted tissues." To be published.

Charalambous, M., Smith, Bennett, Crew, Mackenzie, Ward. (2003). "Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism." Proc. Natl. Acad. Sci. USA. **10**: 1073.

Clarke, A., Hollstein. (2003). "Mouse models with modified p53 sequences to study cancer and ageing." Cell death and differentiation. **10**: 443-450.

Clarke, A., Purdie, Harrison, Morris, Bird, Hooper, Wyllie. (1993). "Thymocyte apoptosis induced by p53-dependent and independent pathways." Nature **362**: 849-852.

Constancia, M., Dean, Lopes, Moore, Kelsey, Reik (2000). "Deletion of a silencer element in Igf2 results in loss of imprinting independent of H19." Nature Genetics **26**: 203-206.

Constancia M., H. M., Hughes J., Dean W., Ferguson-Smith A., Fundele R., Stewert F., Kelsey G., Fowden A., Sibley C., Reik W. (2002). "Placental-specific IGF2 is a major modulator of placental and fetal growth." Nature **417**: 945-948.

Cui, H., Cruz-Correa, Giardiello, Hutcheon, Kafonek, Brandenburg, Wu, He, Powe, Feinburg. (2003). "Loss of IGF2 imprinting: a potential marker of colorectal cancer risk." Science **299**: 1679-1680.

Cui, H., Onyango, Brandenburg, Wu, Hsieh, Feinburg (2002). "Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2." Cancer Res. **62**(22): 6442-6446.

DeChiara, T., Efstratidid and Robertson (1990). "A growth-deficiency phenotype in hetrozygous mice carrying and insulin-like growth factor II gene disrupted by targeting." Nature **345**: 78-80.

DeChiara, T., Robertson and Efstratiadis (1991). "Parental imprinting of the mouse insulin-like growth factor II gene." Cell **64**: 849-859.

Dell, G., Ward, Engstrom (1997). "Regulation of a promoter from the mouse insulin like growth factor II gene by glucocorticoids." FEBS Letters **419**: 161-165.

Donehower, L., Harvey, Slagle, McArthur, Montgomery, Butel, Bradley (1992). "Mice deficient for p53 are developmentally normal but susceptable to spontaneous tumours." Nature **356**: 215-221.

Drewell, R., Brenton, Ainscough, Barton, Hilton, Arney, Dandolo, Surani. (2000). "Deletion of a silencer element disrupts H19 imprinting independently of a DNA methylation epigenetic switch." Development **127**: 3419-3428.

Efstratiadis, A. (1994). "Parental imprinting of autosomal mammalian genes." Curr. Opin. Genat. Dev. **4**: 265.

Efstratiadis, A. (1998). "Genetics of mouse growth." Int J Dev Biol **42**(7): 955-76.

Feil, R., Walter,Allen,Reik (1994). "Developmental control of allelic methylation in the imprinted mouse *Igf2* and *H19* genes." Development **120**: 2933-2943.

Gaudet, F., Hodgson, Eden, Jackson-Grusby, Dausman, Gray, Leonhardt, Jaenisch. (2003). "Induction of tumors in mice by genomic hypomethylation." Science **300**: 442-443.

Granerus, M., Johannisson A., Ekblom P., Engstrom W. (2001). "Insulin-like growth factors I and II induce cell death in Wilms's tumour cells." Mol. Pathol. **54**(1): 30-35.

Griffiths, A., Gelbart, Miller, Lewontin (1999). Modern genetic analysis, W.H. Freeman and company.

Hatada I., O. H., Fukushima Y., Kanetko Y., Inoue M., Komoto Y., Okada A., Ohishi S., Nabetani A., Morisaki H. (1996). "An imprinted gene p57kip2 is mutated in beckwith-Wiedemann syndrome." Nature Genetics **14**: 171-173.

Hseih, C. (1999). "In vivo activity of murine de novo methyltransferases Dnmt3a and Dnmt3b." Mol. Cell. Biol. **19**: 8211-8218.

Hu, J., Pham, Dey, Li, Vu, Hoffman. (2000). "Allele-specific histone acetylation accompanies genomic imprinting of the insulin-like growth factor II receptor gene." Endocrinology **141**(12): 4428-4435.

Jeppesen, P. (1997). "Histone acetylation: a possible mechanism for the inheritance of cell memory at mitosis." Bioessays **19**: 67-74.

Jones, P. (2001). "Cancer: Death and methylation." Nature **409**: 141-144.

Kong F., A. M., Washington M., Keith J., Jirtle R. (2000). "Mp6/Igf2r is mutated in squamous cell carcinoma of the lung." Oncogene **19**(12): 1572-1578.

Lau M., S. C., Liu Z., Bhatt H., Rotwein P., Stewart C. (1994). "Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality." Genes and Development **8**: 2953-2963.

Lee, J., E., J. Pintar, et al. (1990). "Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis." Development **110**(1): 151-9.

Leighton, P., Ingram, Eggenschwiler, Efstratiadis, Tilghman. (1995). "Disruption of imprinting caused by deletion of the H19 gene region in mice." Nature **375**: 34-39.

Li, B., Jaenisch. (1992). "Targeted mutation of the DNA methyltransferase gene results in embryonic lethality." Cell **69**: 915-926.

Li, E., C. Beard, et al. (1993). "Role for DNA methylation in genomic imprinting [see comments]." Nature **366**(6453): 362-5.

Li, T., Vu, Zeng, Nguyen, Hayward, Bonthron, Hu, Hoffman. (2000). "Tissue-specific expression of antisense and sense transcripts at the imprinted Gnas locus." Genomics **1**(69): 295-304.

Loadish, Berk, Zipursky, Matsudaira, Baltimore, Darnell (2000). Molecular cell biology, W.H. Freeman and Company.

Lopes, S., Lewis, Hajkova, Dean, Oswald, Forne, Murrell, Constancia, Bartolomei, Walter, Reik. (2003). "Epigenetic modifications in an imprinting cluster are controlled by a hierarchy of DMRs suggesting long-range chromatin interactions." Hum Mol Genet **12**(3): 295-305.

Matagne, R. F. (1987). "Chloroplast gene transmission in *Chlamydomonas reinhardtii*. A model for its contril by the mating-type locus." Curr. Genet. **12**: 251.

Matzke, M. M., A.J.M. (1993). "genomic imprinting in plants- parental effects and trans-inactivation phenomena." Annu. Rev. Plant Physiol. Plant Mol. Biol., **44**: 53.

Monk, B., Lenhart (1987). "Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ lineages during mouse embryo development." Development **99**: 371-382.

Moore, T., Constancia, Zubair, Bailleul, Feil, Sasaki, Reik. (1997). "Multiple imprinted sense and antisense transcripts, differenatial methylation and tandem repeats in a putative imprinting control region upstream of mouse Igf2." Proc. Natl. Acad. Sci. USA **94**(23): 12509-12514.

Moore, T., Haig,D. (1991). "Genomic imprinting in mammalian development: a parental tug-of-war." Trends Genet. **7**: 45.

Moulton, T., T. Crenshaw, et al. (1994). "Epigenetic lesions at the H19 locus in Wilms' tumour patients." Nat Genet **7**(3): 440-7.

Murrell, A., Heeson, Bowden, Constancia, Dean, Kelsey, Reik (2001). "An intregenic methylated region in the imprinted Igf2 gene augments transcription." EMBO Rep. **12**: 1101-1106.

Okano, B., Haber, Li. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell **99**: 247-257.

Okano, X., Li (1998). "Cloning and characterisation of a family of novel mammalian DNA (cytosine-5) methyltransferases." Nat. Genet. **19**: 219-220.

Ouspenski, I., Hooser, Brinkley. (2003). "Relevance of histone acetylation and replication timing for deposition of centromeric histone CENP-A." Experimental Cell Research **285**: 175-188.

Paulsen, M. (2000). "Sequence conservation and variability of imprinting in the Beckwith-Wiedemann syndrome gene cluster in human and mouse." Hum Mol Genet **9**: 1829-1841.

Paulsen, T., Youngson, Benchaib, Charlier, Segers, Georges, Ferguson-Smith (2001). "Comparitave sequence analysis of the imprinted Dlk1-Gtl2 locus in three mammalian species reveals highly conserved genomic elements and refines comparison with the Igf2-H19 region." Genome Res. **11**: 2085-2094.

Phillips (1999). Techniques in molecular biology.

Reik, W. (2001). "Genomic imprinting: Parental influence on the genome." Nature Reviews **2**: 21-26.

Reik, W., Constancia M. (1997). "Genomic imprinting: Making sense or antisense?" Nature **389**: 669-671.

Rogler, C., Yang, Rossetti (1994). "Altered body composition and increased frequency of diverse malignancies in Igf2 transgenic mice." J. Biol. Chem. **269**: 13779-13784.

Sakai, H., Tsukamoto, Yamamoto, Shirai, Iidaka, Hirata, Yanai, Masegi, Donehower, Tatematsu. (2003). "High susceptibility of nullizygous p53 knockout mice to colorectal tumour induction by 1,2-dimethylhydrazine." J Cancer Res Clin Oncol **129**: 335-340.

Santos, F., Hendrich, Reik, Dean (2002). "Dynamic reprogramming of DNA methylation in the early mouse embryo." Developmental Biology **241**: 172-182.

Sasaki, H., P. A. Jones, et al. (1992). "Parental imprinting: potentially active chromatin of the repressed maternal allele of the mouse insulin-like growth factor II (Igf2) gene." Genes Dev **6**(10): 1843-56.

Sleutels, F., Zwart, Barlow. (2002). "The non-coding Air RNA is required for silencing autosomal imprinted genes." Nature **415**: 810-813.

Spofford, J. B. (1976). "Position effect variegation in Drosophila. In *The genetics and biology of Drosophila* (ed M. Ashburner and E. Novitski)." Academic Press, New York: 955.

Takada, T., Baker, Georgiades, Campell, Freeman, Johnson, Paulsen, Ferguson-Smith (2000). "Delta-like and Gtl2 are reciprocially expressed, differentially methylated linked imprinted genes on mouse chromosome 12." Current Biology **10**: 1135-1138.

Thomas, J. H. (1995). "Genomic imprinting proposed as a surveillance mechanism for chromosome loss." Proc. Natl. Acad. Sci. USA. **92**: 480.

Tricoli, R., Scott, Bell and Shows (1984). "Localization of insulin-like growth factor genes to human chromosomes 11 and 12." Nature **310**: 784.

Varmuza, S., Gilks, C.B. (1993). "Genomic imprinting- defusing the ovarian time bomb." Trends genet., **10**: 346.

Von Mering, C., Bork (2002). "Genome organisation: Teamed up for transcription." Nature **417**: 797-798.

Vu, T., Chuyen, Li, Hoffman. (2003). "Loss of imprinting of Igf2 sense and antisense transcripts in Wilms's tumour." Cancer Research **63**: 1900-1905.

Ward, A., Fisher, Richardson, Pooler, Squire, Bates, Shaposhnikov, Hayward, Thurston and Graham (1994). "Disproportionate growth in mice with Igf2 transgenes." Proc. Natl. Acad. Sci. USA **91**: 10365-10369.

Ward, A., Fisher, Richardson, Pooler, Squire, Bates, Shaposhnikov, Hayward, Thurston and Graham (1997). "Genomic regions regulating imprinting and insulin-like growth factor-II promoter 3 activity in transgenics: novel enhancer and silencer elements." Genes and Function **1**: 25-36.

Webber, L., Tilghman (1998). "The absence of enhancer competition between Igf2 and H19 following transfer into differentiated cells." Mol Cell Biol **18**(4): 1903-1910.

Weksberg, S., Fei, Song, Squire. (1993). "Disruption of insulin-like growth factor two imprinting in Beckwith-Wiedemann syndrome." nature Genetics **5**: 143-150.

Wiedemann (1983). "Tumors and hemihypertrophy associated with Wiedemann-Beckwith syndrome." European Journal pediatrics **14**: 129.

Wollmann, K., Enders, Preece, Ranke (1995). "Growth and symptoms in Silver-Russell syndrome: review on the basis of 386 patients." Eur. J. Pediatr. **154**: 958-968.

Yamada, T., De Souza, Finkelstein, Jirtle. (1997). "Loss of the gene encoding mannose 6-phosphate/insulin-like growth factor II receptor is an early event in liver carcinogenesis." Proc. Natl. Acad. Sci. USA **94**: 10351-10355.

Zwart, S., Wutz, Schinkel, Barlow. (2001). "Bidirectional action of the Igf2r imprint control element on upstream and downstream imprinted genes." Genes and Development **15**: 2361-2366.